Studies on BolA and Ribonuclease R: Two Important Factors in the Control of Bacterial Gene Expression

Ricardo António Neves Moreira

Dissertation presented to obtain the Ph.D degree in Biology

Instituto de Tecnologia Química e Biológica
Universidade Nova de Lisboa

Oeiras, December, 2011

Financial Support from **Fundação para a Ciência e Tecnologia** (**FCT**) – Ph.D: grant - SFRH/BD/36027/2007.

Work performed at:

Control of Gene Expression Laboratory

Instituto de Tecnologia Química e Biológica

Av. da República (EAN)

2781-901 Oeiras – Portugal

Tel: +351-21-4469548

Fax: +351-21-4469549

Supervisor :

Professora Doutora Cecília Maria Pais de Faria de Andrade Arraiano – Investigadora Coordenadora, Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa.
(Head of the Laboratory of Control of Gene Expression, where the work of this Dissertation was performed)

Examiners:

Professora Doutora Paloma López Garcia - Head of Department of Molecular Microbiology & Infection Biology, Centro de Investigaciones Biológicas, Madrid *(Principal Examiner).*

Professora Doutora Leonilde Morais Moreira – Assistant Professor, Instituto Superior Técnico, Universidade Técnica de Lisboa *(Principal Examiner).*

Professora Doutora Maria Teresa Crespo – Principal Investigator, Instituto de Biologia Experimental e Tecnológica, Oeiras.

Professor Doutor António Pedro Alves de Matos - Head of Electron Microscopy Sector of Curry Cabral Hospital, Lisbon.

"Magic is just science we don't understand yet."

-Arthur C. Clarke

Acknowledgments

I would like to thank all the people that directly and indirectly contributed and supported my work, and without whom this PhD dissertation would not have been possible:

Instituto de Tecnologia Química e Biológica (**ITQB**) - the Institution that made this work possible, by providing the facilities and resources. I would like to thank the support of two former ITQB directors **Professor Miguel Teixeira, Professor José Martinho Simões** and the present Director Professor **Luís Paulo Rebelo** for providing the conditions for the presentation of this dissertation.

Fundação para a Ciência e Tecnologia (**FCT**) for the financial support crucial for the accomplishment of this work.

Professor Cecília Arraiano for receiving me in her laboratory and accepting to be my PhD supervisor. Thank you for your support, for your enthusiasm, inspiration and endless capacity to see always the bright side, even in hard and more challenging moments. I also thank you for giving me so many and different opportunities to expand my scientific knowledge participating and presenting my work in several meetings, both in Portugal and abroad.

To all past and present colleagues and friends in the lab for making it such an enjoyable place to be and work at.

Ana – My favorite dance floor partner. Thank you for all the positive energy you transmit, for all your support, ideas and helpful discussions. Thank you for always welcome me even when it was for the most "silly" question.

Filipa – for your friendship, all the funny moments, conversations and also helpful discussions that we had together, inside and outside of the lab.

Andreia Aires – for rescuing me countless times during our so called "emergency" happenings and to help me when I needed.

Cátia Bárria – for making me laugh with the amazing capacity of telling jokes that everyone thinks are real.

Clémentine Dressaire – For all the help, all the knowledge shared and for contributing so much to my work even when far away.

Inês Guinote – for all the long conversations and the lessons about… well… so many things. "Theoretically…" will be the word I will always remember.

Inês Silva – for the enjoyable movies and TV series discussions and all the encouragement during my experimental adventures in the RNA world.

Joana Pissara – for being our audio daily news and for always have an explanation (just like an encyclopedia) when doubt arises.

Zé – for the "laughing out loud" moments at lunch time.

Margarida Matos – for the non-stop good mood. Thanks for your kindness and even for teaching me that I could avoid contaminations in the LB stock by making an aliquot in a falcon tube.

Margarida Saramago – to make me giggle with her daily youtube selection movies.

Michal – for sharing different points of view and ideas work wise.

Patrick – for all the knowledge transferred, all the technics you taught me and all the amazing brainstorming afternoons. Thank you for all your help, support and trust on me.

Rute – for helping when needed and for always be present in the breakfast team for some socializing morning moments.

Sandra – for all the work tips you gave me and the helpful discussions when necessary. For make me laugh so much with your singing skills, mainly with "Magnolia" film soundtrack.

Susana – for the helpful discussions and shared experiences having always a good example (in her dozen's lab books) and making things more practical.
Vânia – For the helpful bioinformatics tips.

To all my colleagues in the other labs in the Institute, specially to the 2008 class of the ITQB PhD course. Thank you for having participated, in one way or another, in this work, by exchanging experiences and friendly words during coffee breaks and dinner parties.

To all my friends – specially to **Alexander, Rita, Zé, Nuno, Ana, Damião** and **Soraia**, for all the comprehension, friendship, support, fun and socializing moments. Thank you for your comments, suggestions and remarks.

To all my family, Mum, Dad, Brother and Sisters, for their eternal support and unconditional love.
To my **Mum and Dad** - for your love, strength, for being present and always curious about what I was doing in the lab.
My **Brother and Sisters** - for being the greatest and call me the family mad scientist – I like it! Thank you for your love and support.

Thank you all!

December 2011

Table of Contents

Abstract

The cellular concentration of a given RNA is the result of the balance between its synthesis and degradation. Both DNA transcription and RNA decay control the final levels of each protein in the cell. BolA is an *Escherichia coli* (*E. coli)* protein, which induces changes in cell morphology when present in high levels. BolA expression is regulated by two different promoters, a sigma 70 (σ^{70}) promoter responsible for the basal levels of this gene in exponential phase and a sigma S (σ^{S}) gearbox promoter important in stress situations and stationary phase of bacterial growth.

The first objective of this PhD work was to further characterize the expression of the *bolA* gene. Based on bioinformatic analysis, we have identified the H-NS protein as a putative transcriptional regulator of BolA. H-NS is a relatively small protein, abundant in bacterial cells and is often compared to eukaryotic histones due to its high affinity for DNA. In order to clarify the possible role of H-NS in BolA transcription, we have constructed an *hns E. coli* mutant. This mutant was compared to the wild type regarding the levels of *bolA* mRNA transcript and *in vitro* DNA-protein interaction studies were performed. These experiments allowed us to demonstrate that H-NS is able to down-regulate the levels of *bolA* mRNA in exponential phase and bind to the *bolA* promoter region. In addition, the DNA-protein interaction studies revealed that H-NS has a special affinity to the curved *bolA* promoter region encompassing both bolA1p and bolA2p promoters.

In the second part of this doctoral project, the aim was to study the specific role of BolA as a transcription factor and characterize its role in cell division and cell shape maintenance. In poor growth conditions, BolA is essential for normal cell morphology in stationary phase and under conditions of

starvation. Previous studies have revealed the influence of BolA in the transcription of different genes involved in cell wall synthesis, such as the mRNA levels of the penicillin binding protein 5 (PBP5) and penicillin binding protein 6 (PBP6). We studied the effect of BolA overexpression in growing cells and showed that this protein can inhibit the cell elongation mechanism. Furthermore, by RT-PCR and dot-blot experiments, we demonstrated that this inhibition is caused by a reduction of *mreB* mRNA levels. Protein levels were also studied, and the effect observed at the mRNA level was reflected in the amount of MreB protein. For the first time BolA has been shown to bind DNA and directly regulate the levels of MreB. MreB filaments are crucial for the bacterial cell cytoskeleton and are essential for the maintenance of a cellular rod shape. The inhibitor effect of BolA on MreB levels might be enough to prevent filament polymerization. Thus, BolA induced morphology is involved in a complex pathway that comprises PBP5, PBP6 and MreB regulation.

The conclusions obtained until this point were quite important for the regulation of a gene with such pleiotropic effects in cell shape maintenance and its functional characterization. To pursue our task of understanding the role of BolA as a new transcription factor, we performed microarrays to study the global effect of BolA in *E. coli* transcription regulation. Our results displayed a great variety of genes affected by the presence of BolA in the cell. These genes are related not just with cell morphology but also with cell metabolism, cell motility and stress response. Among the stress response genes, sigma E (σ^E) was the unique polymerase subunit to be significantly affected at the mRNA level by BolA. In *E. coli* one of the key pathways involved in maintaining cell envelope integrity during stress and normal growth is controlled by σ^E. The regulation of this sigma factor by BolA was assayed by northern blot and confirmed. It is known that sigma σ^E is involved in the regulation of at least three important *E.*

coli small RNAs (sRNAs) that control different outer membrane porins (OMPs) during the envelope stress response. In order to establish a relationship between the BolA properties to alter the OmpC/OmpF ratio, we analyzed the possibility of OMP-related sRNAs being affected. Our results showed several sRNAs varying with the BolA presence during the exponential growth of cells. From the three sRNAs regulated by σ^E, RybB and CyaR were both overexpressed when the cells grow in the presence of elevated BolA. Moreover, the levels of six other sRNAs were also affected, either negatively or positively. The mechanism of regulation is still not clear. However, preliminary experiments showed that apparently, BolA is not influencing sRNAs stability, and probably the regulation is at the transcription level.

Although transcription is quite important to determine steady-state levels of a given mRNA, post-transcriptional control is critical in the regulation of gene expression. To finalize this doctoral study I also dedicated some time performing studies on the post-transcriptional regulation of gene expression. RNases are the enzymes that intervene in the processing, degradation and quality control of all types of RNAs. RNase R is a processive 3'-5' exoribonuclease that belongs to the RNase II family of enzymes that is expressed in high amounts when cells are faced with a stress challenge. This protein has been implicated in the virulence mechanisms of different pathogenic organisms. In *Streptococcus pneumoniae* (*S. pneumoniae*), there is a unique homologue of the RNase II family of enzymes that was shown to be a RNase R-like protein. We challenged cells growing in different temperatures, and we observed that RNase R responds to cold shock, increasing its mRNA and protein levels. Analysis of the *S. pneumoniae* genome showed that RNase R is upstream and overlapping with the *smpB* gene open reading frame. The latest observation gave rise to studies based on the relationship between these two partners in the cell surveillance system called trans-translation. This

quality control mechanism targets the degradation of mRNAs without a stop codon, their respective abnormal proteins and rescues the stalled ribosomes. In collaboration with Doctor Paloma López (Centro de Investigaciones Biológicas, Madrid) and Doctor Mónica Amblar (Centro Nacional de Microbiología, Instituto de Salud Carlos III, Madrid) we have constructed *S. penumoniae* RNase R and SmpB mutants and studied the possible inter-regulation of these two partners. Our results showed that RNase R protein levels are dependent on the SmpB and vice-versa. Furthermore, the *smpB* mRNA levels were significantly higher in the *rnr* mutant strain, indicating a possible role of this ribonuclease in the degradation of *smpB* transcript.

The work of this dissertation further characterized the role of *E. coli* BolA protein in terms of its complex network of regulation and on its influence in cell morphology and envelope maintenance. Moreover, the characterization of RNase R and its possible impact in the trans-translation process can be applied to better understand its role in gram-positive human pathogens such as *S. pneumoniae.*

Resumo

A concentração celular de RNA é o resultado de um balanço entre a sua transcrição e a sua degradação. Estes dois processos permitem controlar o nível de proteína final na célula. A proteína BolA de *Escherichia coli* (*E. coli*) induz modificações na morfologia ceuluar quando presente em grandes quantidades. A nível transcripcional, a expressão do seu gene é regulada por dois promotores. A expressão basal deste gene na fase exponencial de crescimento é controlada pela proteina sigma 70 (σ^{70}). Em situações de stress ou em fase estacionária do crescimento a proteína sigma S (σ^{S}) é responsável pelos seus níveis de expressão a partir de um promotor "gearbox".

O primeiro objectivo deste trabalho foi a continuação da caracterização da expressão do gene *bolA*. Com base em análises bioinformáticas, identificámos um possível regulador da expressão do gene *bolA,* a proteína H-NS. Esta é uma proteína relativamente pequena, abundante nas células bacterianas e frequentemente comparada com as histonas de células eucariotas devido à sua grande afinidade para moléculas de DNA. De forma a estudar o possível efeito da proteína H-NS na transcrição do gene *bolA* em *E. coli,* construímos um mutante desta proteína e comparámos os níveis de RNA mensageiro do gene *bolA* entre este mutante e a correspondente estirpe selvagem. Adicionalmente foram também efectuados estudos *in vitro* de forma a determinar a interacção DNA-proteína. Os resultados obtidos permitiram demonstrar que em fase exponencial a proteína H-NS se liga à região promotora do gene *bolA* reduzindo a sua expressão. Além do mais, as interacções DNA-proteína revelaram que a proteína H-NS tem uma grande afinidade para a região reguladora a montante do gene *bolA* que engloba os dois promotores responsáveis pela sua transcrição, região esta que por análise bioinformática apresenta uma curvatura.

Na segunda parte deste trabalho, o objectivo foi estudar a função da proteína BolA como factor de transcrição. Em condições de crescimento não favoráveis, como por exemplo fase estacionária, a proteína BolA é essencial para manter a morfologia das células. Estudos anteriores mostraram ainda a influência desta proteína na expressão de vários genes cujos produtos estão envolvidos na síntese da parede celular, tais como os genes *dacA* e *dacC* que codificam para as proteínas PBP5 e PBP6. Neste contexto, estudámos o efeito da sobre-expressão da proteína BolA durante o crescimento celular e mostrámos que esta proteína inibe o mecanismo de alongamento celular. Adicionalmente, utilizando RT-PCR e RNA dot-blot, demonstrámos que esta inibição é causada pela redução dos níveis de RNA do gene *mreB*. Os níveis celulares da proteína MreB foram também analisados e o efeito observado ao nível do RNA reflecte-se também nos níveis de proteína detectada. Foi demonstrado pela primeira vez que a proteína BolA tem a capacidade de se ligar ao DNA e acima de tudo, que é um regulador da transcrição do gene *mreB*. A proteína MreB é caracterizada por se polimerizar em forma de hélice distribuida ao longo da célula, sendo uma proteína essencial para a manutenção da forma de bacilo. O efeito inibidor da proteína BolA nos níveis da proteína MreB podem ser assim suficientes para a prevenção da formação dos filamentos. Concluiu-se então que as alterações morfológicas causadas pela proteína BolA deverão envolver uma complexa rede que compreende as proteínas PBP5, PBP6 e MreB.

As conclusões obtidas até este ponto foram de elevada relevância para a compreensão da função de uma proteína com variados efeitos na morfologia celular. De forma a prosseguir os estudos relativos à função da proteína BolA como factor de transcrição, realizámos estudos de transcriptómica para avaliar o seu efeito global na célula. Os resultados obtidos foram surpreendentes e mostraram que este regulador afecta a expressão de uma grande quantidade de

genes. Esses genes não estão somente relacionados com morfologia celular, mas também com o metabolismo, a motilidade e a resposta a stress. De todos os factores sigma envolvidos na resposta a stress, a proteína sigma E (σ^E) foi a única que se observou estar significativamente afectada a nível transcricional pela presença da proteína BolA. É sabido que em *E. coli* uma das vias mais importantes na manutanção da integridade da parede celular é controlada por σ^E. Os efeitos observados a nível da regulação da expressão desta sub-unidade da polimerase foram analisados e confirmados por northern blot. Adicionalmente, sabe-se que a proteína σ^E está envolvida na regulação da expressão de pelo menos três pequenos RNAs (sRNAs) que estão envolvidos no controlo da expressão de diferentes proteínas da membrana externa (OMPs) em situações de stress relacionado com a membrana celular. De forma a estabelecer uma relação entre a proteína BolA e as alterações no rácio OmpC/OmpF anteriormente observadas, analisou-se a possibilidade dos sRNAs relacionados com a expressão das OMPs estarem a ser afectados a nível transcripcional. Os resultados obtidos mostraram que na presença da proteína BolA em fase exponencial, há oito sRNAs distintos regulados a nível transcripcional. Dos três que se sabem ser regulados pela proteína σ^E, RybB e CyaR foram aqueles que se observou apresentarem maior variação na presença de uma elevada concentração de BolA. O mecanismo de regulação pelo qual a proteína BolA afecta a expressão dos sRNAs é desconhecido. Contudo, resultados preliminares mostram que a proteína BolA não parece afectar a estabilidade dos sRNAs e que provavelmente estará a ter um papel fundamental no controlo da transcrição dos mesmos.

Embora o controlo da transcrição seja muito importante para os níveis de mRNA na célula, o controlo pós-transcripcional desempenha também um papel critico nos níveis finais de mRNA. A parte final desta dissertação foi dedicada à realização de estudos pós-transcripcionais da expressão génica. As RNases são

enzimas que intervêm no controlo da degradação, processamento e controlo de qualidade de todos os tipos de RNAs na célula. A proteína RNase R, uma ribonuclease da família da proteína Rnase II, está caracterizada como sendo capaz de degradar RNAs estruturados no sentido 3'-5' sendo expressa em maior quantidade situações de stress. Esta proteína está ainda envolvida em mecanismos de virulência de diferentes agentes patogénicos. Em *Streptococcus pneumoniae* (*S. pneumoniae*), foi recentemente descrita uma proteína homóloga estruturalmente e bioquimicamente muito semelhante à RNase R de *E. coli*. De forma a prosseguir com os estudos efectuados sobre esta enzima, diferentes culturas de células de *S. pneumoniae* foram sujeitas a diferentes temperaturas observando-se, tal como em *E. coli*, um aumento dos níveis de mRNA que codifica esta enzima, assim como os níveis da expressão da proteína. Adicionalmente, por análise bioinformática do genoma de *S. pneumoniae*, observou-se que neste organismo, a proteína RNase R está localizada a montante do gene *smpB* e a sua extremidade 3' sobrepõem-se com a extremidade 5' desse mesmo gene. Sendo que estes dois genes codificam para duas proteínas importantes envolvidas no mecanismo de controlo de qualidade "trans-translation", o possível significado biológico da sobreposição destes dois genes foi alvo de estudo. O mecanismo "trans-translation" está envolvido na degradação de mRNAs abrerrantes, como por exemplo, mRNAs que não possuem codão stop, e libertação dos respectivos ribossomas que ficam impedidos de continuar a tradução dos mRNAs. Em colaboração com a Dra. Paloma López (Centro de Investigaciones Biológicas, Madrid) e Dra. Mónica Amblar (Centro Nacional de Microbiologia, Instituto de Salud Carlos III, Madrid), construímos mutantes das proteínas RNase R e SmpB de *S. pneumoniae* e estudámos a possível inter-regulação destes dois parceiros. Os resultados obtidos mostraram que os níveis da proteína RNase R são dependentes da proteína SmpB e vice-versa. Adicionalmente, observou-se que os níveis de mRNA de *smpB* são

significativamente mais elevados numa estirpe mutante do gene *rnr*, indicando uma possível função desta ribonuclease na degradação do transcrito *smpB*.

O trabalho descrito nesta dissertação contribuíu para a caracterização da proteína BolA de *E. coli* relativamente à sua complexa rede de regulação, respectiva influência na morfologia celular e manutenção da homeostase da membrana celular. Alvo deste estudo foi também a caracterização e análise do envolvimento da RNase R de *S. pneumoniae* na regulação da expressão do gene *smpB,* que codifica para uma proteína importante no mecanismo "trans-translation" permitindo desta forma progredir no conhecimento do papel desta RNase neste agente patogénico humano.

List of Publications

Moreira, R.N., C. Dressaire, and C.M. Arraiano. 2011. Multiple target regulation by BolA: The impact on stress related small non-coding RNAs in *Escherichia coli*. **Manuscript in preparation**

Guinote I.B., R.N. **Moreira**, P. Freire, C.M. Arraiano. 2011 Characterization of the BolA homologue IbaG: a new gene involved in acid resistance. **Submitted to J Microbiol Biotechnol**

Moreira R.N.*, S. Domingues*, S.C. Viegas, C.M. Arraiano. 2011 Criss-cross regulation of RNase R and SmpB in *Streptococcus pneumoniae*. **Submitted to J. Mol. Biol.***- Authors contributed equally

Guinote, I.B., **R.N. Moreira**, P. Freire, M. Vicente, C.M. Arraiano. 2011 Gram-negative cell wall regulation and BolA mediated protection against stresses. **World J Microbiol Biotechnol.** Accepted

Moreira, R.N., C. Dressaire, S. Domingues, and C.M. Arraiano. 2011. A new target for an old regulator: H-NS represses transcription of bolA morphogene by direct binding to both promoters. **Biochem Biophys Res Commun. 411:50-55**

Arraiano, C.M., J.M. Andrade, S. Domingues, I.B. Guinote, M. Malecki, R.G. Matos, **R.N. Moreira**, V. Pobre, F.P. Reis, M. Saramago, I.J. Silva, and S.C. Viegas. 2010. The critical role of RNA processing and degradation in the control of gene expression. **FEMS Microbiol Rev. 34:883-923**

Freire, P., **R.N. Moreira**, and C.M. Arraiano. 2009. BolA inhibits cell elongation and regulates MreB expression levels**. J Mol Biol. 385:1345-1351**

Dissertation Outline

This Dissertation is divided into six chapters.

Chapter one consists of a general introduction on transcription and post-transcriptional regulation of gene expression. A particular emphasis is given to BolA, small non-coding RNAs and RNase R, the main focus of this Dissertation.

Chapter two consists of an article published in the *Biochemical and Biophysical Research Communications* in which the author of this dissertation played a major contribution. A mutant of *hns* was constructed and the influence of H-NS protein in *bolA* transcription was studied, leading to important conclusions regarding the regulation of this *E. coli* morphogene.

In chapter three the role of BolA as a transcription factor and the characterization of its role in the cell division and cell shape maintenance were analysed. We studied the effect of BolA overexpression in growing cells and showed that this protein acts as a transcription factor and inhibits cell elongation mechanism. This chapter is presented as a manuscript that was published in the *Journal of Molecular Biology*.

Chapter four is centred in the newly found characteristic of BolA as a transcription factor. We performed microarrays to study the global effect of BolA in *E. coli* transcription regulation. We showed that the stress sigma factor E and outer membrane proteins related sRNAs are affected by BolA in exponential phase of cell's growth and discuss the importance of this regulation.

Chapter five focuses on the post-transcriptional regulation of gene expression in the human pathogen bacteria *Streptococcus pneumoniae.* The main focus of this chapter was to study the expression of RNase R in cold-shock and its involvement in the trans-translation system. This chapter was submitted to *Journal of Molecular Biology*.

To finalize, Chapter six is the final discussion that brings together all the conclusions from the other chapters and proposes future perspectives.

Abbreviations

A adenine

Amp ampicillin

ATP adenosine triphosphate

bp base pair

BSA bovin serum albumine

B. subtillis *Bacillus subtillis*

°C degree Celsius

C citosine

CDS cold shock domain

Cm chloramphenicol

cpm counts per minute

Δ deletion

Da dalton

DTT dithiothreitol

dATP 2′-deoxyadenosine 5′-triphosphate

DNA deoxyribonucleic acid

DNase deoxyribonuclease

dsDNA double stranded DNA

dsRNA double stranded RNA

E. coli *Escherichia coli*

EDTA ethylenediaminetetraacetic acid

EF-Tu elongation factor TU

EMSA electrophoretic mobility shift assay

G guanine

g relative centrifugal force

GFP green fluorescence protein

Glu glutamate

h hour

His histidine

H-NS histone-like nucleoid structuring protein

HU heat unstable protein

IHF integration host factor

IPTG IsoPropyl-β-D-thiogalactopyranoside

Kan kanamycin

Kb kilobase

K_D dissociation constant

kDa kilodalton

L liter

LB luria- bertani broth

Log logarithm

M molar/ molarity (mol/L)

mg milligram

µg microgram

µl microliter

µM micromolar

Mg magensuim

ml milliliter

min minute

mM milliMolar

mmol millimole

Mol mole

Mr molecular mass

mRNA messenger RNA

ng nanogram

MW molecular weight

nM nanoMolar

nmol nanomole

nt nucleotide

OD optical density

Oligo oligonucleotide

o.n. over night

ORF open reading frame

^{32}P phosphorus 32 radionucleotide

PAA polyacrylamide

PAGE polyacrylamide gel electrophoresis

PAP I Poly(A) Polymerase I

PBP penicillin binding protein

PBS phosphate-buffered saline buffer

PCR polymerase chain reaction

pmol picomol

PNPase polynucleotide phosphorylase

Poly(A) polyadenylate

psi pressure unit

RBS ribosome binding site

rcf relative centrifugal force

RNA ribonucleic acid

RNase ribonuclease

rpm rotations per minute

rRNA ribosomal RNA

RT reverse transcriptase

RT-PCR reverse transcriptase polymerase chain reaction

s second

SDS sodium dodecyl sulfate

SOC SOB ("Super Optimal Broth") with glucose

S. pneumoniae Streptococcus pneumoniae

sRNA small RNA

SSC sodium cholride/ sodium citrate

ssDNA single stranded DNA

ssRNA single stranded RNA

StpA suppressor of td- phenotype A

T tymine

Tet tetracycline

Thy thymine

tmRNA transfer messenger RNA

Tris trishydroxymethylaminomethane (2-Amino-2-(hydroxymethyl)propane-1,3-diol**)**

tRNA transfer RNA

U uracil

UTP uracil triphosphate

UV ultraviolet radiation

V volt

vol volume

v/v volume/volume

wt wild-type

w/v weight/volume

Chapter 1

Introduction

Part of this chapter was based on:

Guinote, I.B., **R.N. Moreira**, P. Freire, M. Vicente, C.M. Arraiano. 2011 Gram-negative cell wall regulation and BolA mediated protection against stresses. World J Microbiol Biotechnol. Accepted

and

Arraiano, C.M., J.M. Andrade, S. Domingues, I.B. Guinote, M. Malecki, R.G. Matos, **R.N. Moreira**, V. Pobre, F.P. Reis, M. Saramago, I.J. Silva, and S.C. Viegas. 2010. The critical role of RNA processing and degradation in the control of gene expression. FEMS Microbiol Rev. 34:883-923

Introduction

Environmental changes induce adaptive cellular responses that can lead to alterations in the genetic expression and physiology of the cell. These modifications allow the cell to survive and adapt to the new environment. Regulation of transcription allows controlling the concentration of an RNA molecule during its synthesis. Gene products are conserved among different species of bacteria and the extraordinary variety of bacterial life results from differences in the relative amounts of these products and in the timing of their expression. Regulation can occur at every step on the pathway to gene expression (Browning and Busby, 2004). mRNAs vary greatly in stability and alterations in mRNA decay have a very high impact in cellular processes. The cellular concentration of a given RNA is the result of the balance between its synthesis and degradation. Both transcription and decay control the levels of each protein in the cell. Regulating gene expression at the messenger level is of utmost importance to give adaptability in the context of the small genome size found in prokaryotes where transcription and translation are coupled.

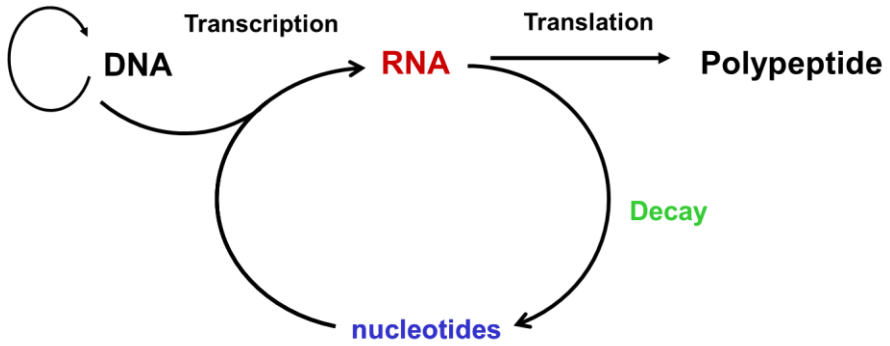

Fig. 1. Schematic representation of transcriptional and post-transcriptional control of gene expression in bacteria. In a first step DNA is transcribed in RNA. The new synthetized RNA can be translated in a protein or be degraded in nucleotides that can be used by the cell. The cellular level of any given RNA/protein is a function of both its rate degradation as well as its rate of synthesis. RNA will be the object of study in this work.

Transcriptional Regulation

The central component in transcriptional regulation in bacteria is the multi-subunit DNA-dependent RNA polymerase, which is responsible for all transcription (FIG. 2) (Browning and Busby, 2004; Ebright, 2000; Murakami et al., 2002). In bacteria, RNA polymerase exists in two different states. One form, known as the core enzyme, can catalyse RNA synthesis but is unable to bind to the promoters in the DNA sequence. The second form of RNA polymerase, the holoenzyme, is capable of both RNA synthesis and promoter recognition.

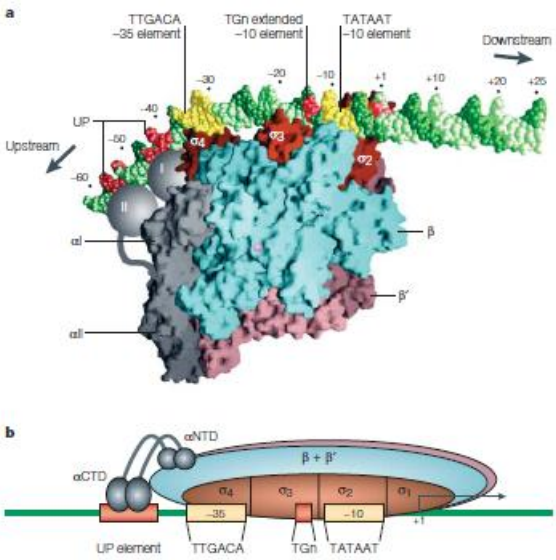

Fig. 2. RNA polymerase and its interactions at promoters. (a) A model based on crystallographic studies of the RNA polymerase holoenzyme docking to a promoter. The DNA strands are shown in green, with the –10 and –35 elements highlighted in yellow and the TGn extended –10 and the UP elements highlighted in red. b - A cartoon illustration of the model shown in part (a), showing the different interactions between promoter elements and the RNA polymerase. The consensus sequences for the -35 (TTGACA), extended –10 (TGn) and -10 (TATAAT) elements are shown. Adapted from (Browning and Busby, 2004)

The RNA polymerase holoenzyme contains an additional subunit, σ, giving the complex three main functions: to ensure the recognition of specific promoter sequences; to position the RNA polymerase holoenzyme at a target promoter; and to facilitate unwinding of the DNA duplex near the transcript start site (Wosten, 1998). Seven sigma factors have been identified in *E. coli* (Gross et al., 1998;

Helmann and Chamberlin, 1988; Ishihama, 1997) and each σ subunit is required by RNA polymerase in order to transcribe specific set of genes.

Promoters control the transcription of all genes. Transcription initiation requires the interaction of RNA polymerase with promoter DNA and the formation of an open complex, in which the duplex DNA around the transcript start-point is unwound (FIG. 2b) (deHaseth et al., 1998). Promoters recognized by sigma contain two well-characterized elements, the -10 and -35 promoter sequences, which are located 10 and 35 base pairs upstream of the transcription start site. These elements specify the initial binding of RNA polymerase to a promoter, but the relative contribution of each element differs from promoter to promoter. As the role of these promoter elements seems to be primarily to dock the RNA polymerase, deficiencies in one element can be compensated by another. The amount of free RNA polymerase that is available in the cell is limited (Ishihama, 2000). Additionally, the supply of σ factors is limited, so there is intense competition between different promoters for RNA polymerase holoenzyme (Ishihama, 2000; Maeda et al., 2000). Five distinct molecular mechanisms seem to ensure the prudent distribution of RNA polymerase between competing promoters. These involve promoter DNA sequences, σ factors, small ligands, transcription factors and the folded bacterial chromosome structure. Some promoters are active in the absence of additional factors and when the genes under their control are not required, they are silenced by transcription repressors (Martinez-Antonio and Collado-Vides, 2003). The *E. coli* genome contains more than 300 genes that encode proteins that are predicted to bind to promoters, and to either up- or downregulate transcription (Madan Babu and Teichmann, 2003; Perez-Rueda and Collado-Vides, 2000).

Transcription activators

Three general mechanisms are described for transcription activation (FIG. 3). In Class I (FIG. 3a), the activator binds to a target that is located upstream of the promoter –35 element and recruits RNA polymerase to the promoter by directly interacting with the RNA polymerase αCTD. In Class II activation (FIG. 3b), the activator binds to a target that overlaps the promoter –35 element and contacts domain 4 of the RNA polymerase σ subunit (Dove et al., 2003). This

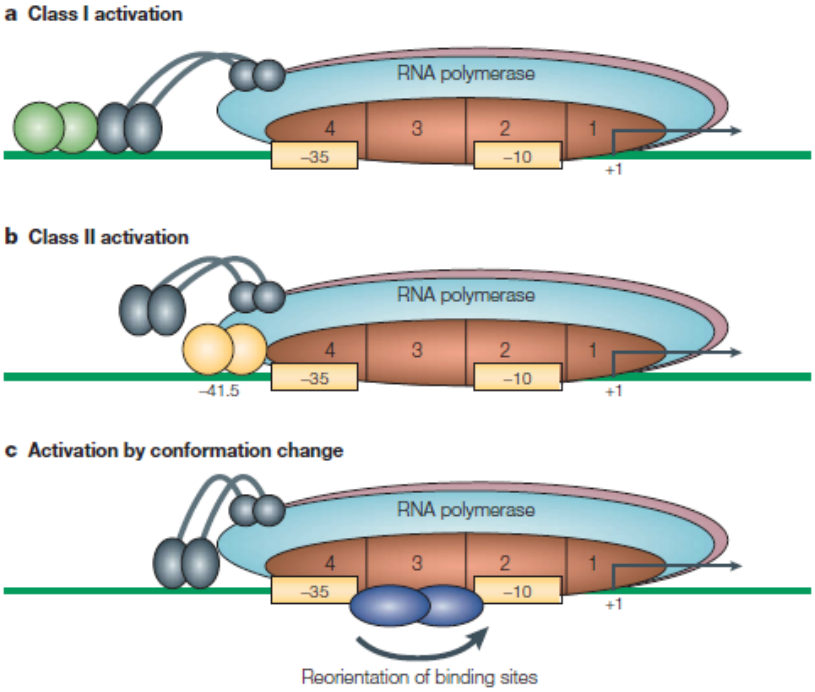

Fig. 3. Many activators function as dimers, and are shown as dimers here. Interacting proteins are shown adjacent to each other. (a) Class I activation. The activator is bound to an upstream site and contacts the αCTD of RNA polymerase recruiting the polymerase to the promoter. (b) Class II activation. The activator binds to a target that is adjacent to the promoter –35 element, and the bound activator interacts with domain 4 of σ^{70}. (c) Activation by conformation changes. The activator (shown in blue) binds at, or near to, the promoter elements so that the RNA polymerase holoenzyme can bind to the promoter. Adapted from (Browning and Busby, 2004)

contact also results in recruitment of RNA polymerase to the promoter, but other steps in initiation can also be affected. The third mechanism for simple activation is found in cases where the activator alters the conformation to enable the interaction of RNA polymerase with the promoter -10 and/or –35 elements. This requires the activator to bind at, or very near to, the promoter elements (FIG 3c).

Transcription repressors

Repressor proteins reduce transcription initiation at target promoters (FIG. 4). Steric hindrance of RNA polymerase binding to promoter DNA is probably the simplest mechanism of repression (FIG. 4a). The repressor binding site is located in, or close to, the core promoter elements. However, in some cases, the

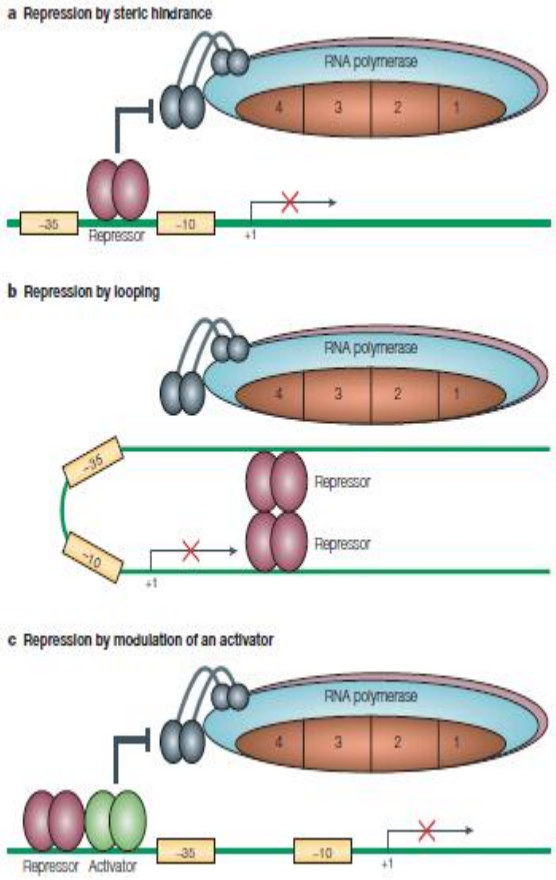

Fig. 4. Mechanisms of repression. (a) Repression by steric hindrance. The repressor binding site overlaps core promoter elements and blocks recognition of the promoter by the RNA polymerase holoenzyme. (b) Repression by looping. Repressors bind to distal sites and interact by looping, repressing the intervening promoter. (c) Repression by the modulation of an activator protein. The repressor binds to an activator and prevents the activator from functioning by blocking promoter recognition by the RNA polymerase holoenzyme. Adapted from (Browning and Busby, 2004)

repressor might not prevent binding of RNA polymerase to the promoter, but instead might interfere with post-recruitment steps in transcription initiation (Muller-Hill, 1998). At other promoters, multiple repressor molecules bind to promoter-distal sites, and repression might be caused by DNA looping, which shuts off transcription initiation in the looped domain (FIG. 4b). Finally, complex cases have been found where the repressor functions as an anti-activator (FIG. 4c).

Folded chromosomes and transcription

In addition to RNA polymerase and transcription factors, the chromosome of bacteria cells is bound by a battery of proteins involved in DNA repair, replication, protection, and folding. The folded *E. coli* chromosome is called the nucleoid and proteins involved in folding the chromosome are known as nucleoid-associated proteins. In *E. coli*, panoply of proteins are involved in this compaction, including Fis, IHF, H-NS and HU, StpA (an H-NS homologue) and Dps. These so-called nucleoid proteins are abundant in the cell, although the concentrations of some fluctuate sharply depending on the growth conditions. The binding of these nucleoid proteins to DNA, and the resulting folding of the bacterial chromosome, must affect the distribution of RNA polymerase between promoters. The effects of these proteins have been unravelled on a case-by-case basis at individual promoters. H-NS (Histone-like protein) protein is one of the most studied nucleoid associated proteins and was seen as a protein that can completely silence gene expression by forming extended nucleoprotein structures (Jordi and Higgins, 2000; Petersen et al., 2002; Schnetz, 1995). Interestingly, in *E. coli*, it has been found that many locations where H-NS is bound to the DNA also contain RNA polymerase (Grainger et al., 2006; Oshima et al., 2006) perhaps suggesting trapping of RNA polymerase may be a common mechanism of

transcription regulation by H-NS. Nucleoid associated factors can also work together to influence transcription, causing activation or repression, depending on the context of their binding sites (McLeod and Johnson, 2001).

Overall, transcription is a very important mechanism in the cell and being a very complex process it needs to be tightly regulated. Part of this thesis is focused on the transcriptional studies of *bolA* gene. The first part will give emphasis to the transcriptional regulation of this *E. coli* morphogene. In a second part, the hypothesis of BolA protein acting in the cell as a transcriptional factor is analysed.

bolA morphogene and transcriptional regulation in *E. coli*

The gene *bolA* was first described as a stationary phase gene (Aldea et al., 1989; Aldea et al., 1990). The expression of *bolA* is growth phase-regulated and controlled by the sigma S (σ^S) (Lange and Hengge-Aronis, 1991). However, *bolA* has also been established as a general stress response gene induced during exponential phase in response to several stresses (Santos et al., 1999). The overexpression of *bolA* leads to substantial changes in the cell and the bacterial bacilli transform into spheres. This effect of *bolA* on cell morphology is mediated by the induction of penicillin binding protein 5 (PBP5) and penicillin binding protein 6 (PBP6) D,D-carboxypeptidases (Aldea et al., 1988; Guinote et al., 2011; Santos et al., 2002). *Escherichia coli* BolA protein contains one defined BolA/YrbA domain with potential for DNA-binding and regulatory activity. It was already shown that BolA can bind to the *dacA* and *dacC* promoter regions and upregulate these genes, hence expanding its potential as a transcriptional regulator, with activator abilities. Moreover, *bolA* gene increased expression was shown to be an important inducer of biofilm formation (Vieira et al., 2004) and to modulate cell

permeability (Freire et al., 2006b). As a result BolA constitutes a privileged target to study molecular mechanisms of adaptation of *Escherichia coli* when facing adverse growth conditions.

bolA regulatory network

bolA encodes a protein with a predicted molecular weight of 13,5 KDa and is transcribed in a clockwise direction in the *E. coli* chromosome. This gene has two different upstream promoters, a P1 promoter, under the control of σ^S and an upstream P2 promoter, controlled by σ^{70}. (FIG. 5) Moreover, *bolA* mRNA transcribed by P2 is always detected along bacterial growth, in low amounts, being classified as a weak and constitutive promoter (Aldea et al., 1989; Lange and Hengge-Aronis, 1991). Nevertheless, transcript originated by P1 promoter is primarily detected when cells are already in late-exponential phase of growth, entrance to the stationary phase or upon stress conditions with a concomitant decrease of bolA P2 levels (Aldea et al., 1989). This observation gave rise to a promoter occlusion hypothesis stating that the physical interaction of the trans-

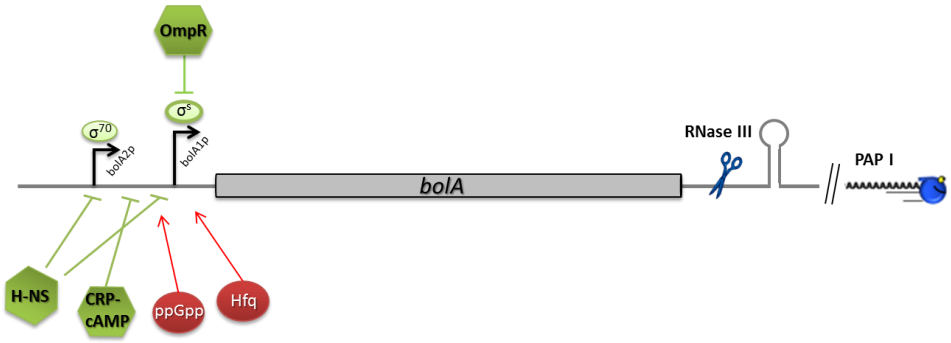

Fig. 5. *bolA* transcriptional and post-transcriptional regulation. At the transcriptional level, *bolA* expression is negatively regulated by OmpR, H-NS, CRP-cAMP and positively regulated by ppGpp and PAPI. At post-transcriptional level, RNase III and Hfq positively affect *bolA* mRNA stability.

-cription machinery by σ^S at the P1 promoter might interfere with the ability of RNA polymerase to either interact or proceed from P2. P1 promoter is growth phase and growth rate regulated and contains a gearbox element characteristic of several other structural genes. Additionally, it was also observed that P1, similarly to σ^S, is down regulated by cAMP levels (Lange and Hengge-Aronis, 1991). At transcriptional level, Ribonuclease III (RNase III) has an important role in the expression of the *bolA* gene (Freire et al., 2006a; Santos et al., 1997). Also polyadenilation, which inversely correlates with bacterial growth rate (Jasiecki and Wegrzyn, 2003), reduces RssB-mediated ClpXP σ^S proteolysis, increasing rpoS protein levels, thus contributing for the transcription of σ^S dependent genes like *bolA* (Santos et al., 2006). Additionally, Yamamoto and co-workers, saw in vitro that *bolAp1* is negatively regulated by phosphor-OmpR (Yamamoto, 2000).

At post-transcriptional level, under carbon starvation, it was shown that RNase III is also involved in the *bolAp1* stability (Freire et al., 2006a). The *bolAp1* mRNA is induced nine-fold in a WT strain while in an *rnc* mutant strain is four-fold induced, showing a decrease in *bolAp1* RNA in the absence of RNase III. Taking together this data, RNase III was classified as a contributor to the stability of *bolAp1* mRNA, thus correlating with its role in *rpoS* RNA.

As it can be observed in the example given above, *bolA* transcriptional regulation is a complex network where different proteins or transcriptional factors are involved. The expression of a gene depends not only on the rate of synthesis of its RNA in the cell (transcription) but also on its rate of decay. Although transcription is quite important to determine steady-state levels, post-transcriptional control is critical in the regulation of gene expression.

Post-Transcriptional Regulation

RNA degradation plays a fundamental role in all biological processes, since the fast turnover of mRNA permits rapid changes in the pattern of gene expression. mRNAs vary greatly in stability, and alterations in mRNA decay have a very high impact in cellular processes. The balance between mRNA degradation and mRNA synthesis determines the intracellular levels of individual mRNAs in the cells. There are two very well studied and major post-transcriptional groups of regulators in bacteria: small regulatory RNAs (sRNAs) and Ribonucleases (RNases).

Regulatory RNAs can modulate transcription, translation, mRNA stability, and DNA maintenance or silencing. This panoply of events occur through a variety of mechanisms, such as changes in RNA conformation, protein binding, base pairing with other RNAs, and interactions with DNA (Waters and Storz, 2009). sRNAs have been identified in a wide range of bacteria. The major families of sRNAs include antisense RNAs, synthesized from the strand complementary to the mRNA they regulate, trans encoded sRNAs that act by limited complementarity with their targets, and sRNAs that regulate proteins by binding to and affecting protein activity (Gottesman and Storz, 2010).

sRNAs have been extensively studied over the last years because of their high importance in the post-transcriptional regulation of bacterial gene expression.

In the third part of this dissertation, we will study the direct/indirect role of BolA in the transcription of different sRNAs of *Escherichia coli*.

Small regulatory RNAs

sRNAs from enterobacterial species are usually of ~ 50 to 200 nucleotides, often expressed under specific growth, stress or virulence conditions (Papenfort and Vogel, 2009). Whilst some sRNAs modulate the activity of proteins (Gottesman and Storz, 2010), most of the characterized sRNAs regulate gene expression by base pairing with mRNAs and those can be split into two different classes: the ones having perfect base pairing with their target RNA and those with more limited complementarity. For the first class, one of their functions is to allow the directed cleavage of the mRNA encoded on the opposite strand. However, the most prevalent role for antisense sRNAs in bacteria has been the repression of genes that encode toxic proteins, functionally working as a toxin/anti-toxin system (Fozo et al., 2008).

The second class of base pairing sRNAs is the trans-encoded sRNAs, which share only limited complementarity with their target mRNAs and are normally induced under stress conditions. These regulators modulate the translation and/or stability of target mRNAs mainly in a negative manner (Aiba, 2007). The contact between the sRNA and its target mRNA usually leads to repression of protein levels through translational inhibition and/or mRNA degradation. However, sRNAs can also activate expression of their target mRNAs through an anti-antisense mechanism whereby base pairing of the sRNA disrupts an inhibitory secondary structure (Prevost et al., 2007). The RNA chaperone Hfq is, in many cases, required for trans-encoded sRNA-mediated regulation, most probably by actively remodeling the interacting RNAs to melt secondary structures or by indirectly increase the local RNA concentrations by bringing together sRNAs and target mRNAs (Valentin-Hansen et al., 2004).

Trans-encoded sRNA typically has multiple target mRNAs (Fig. 6) (Papenfort and Vogel, 2009). Since this short RNAs are usually associated with a

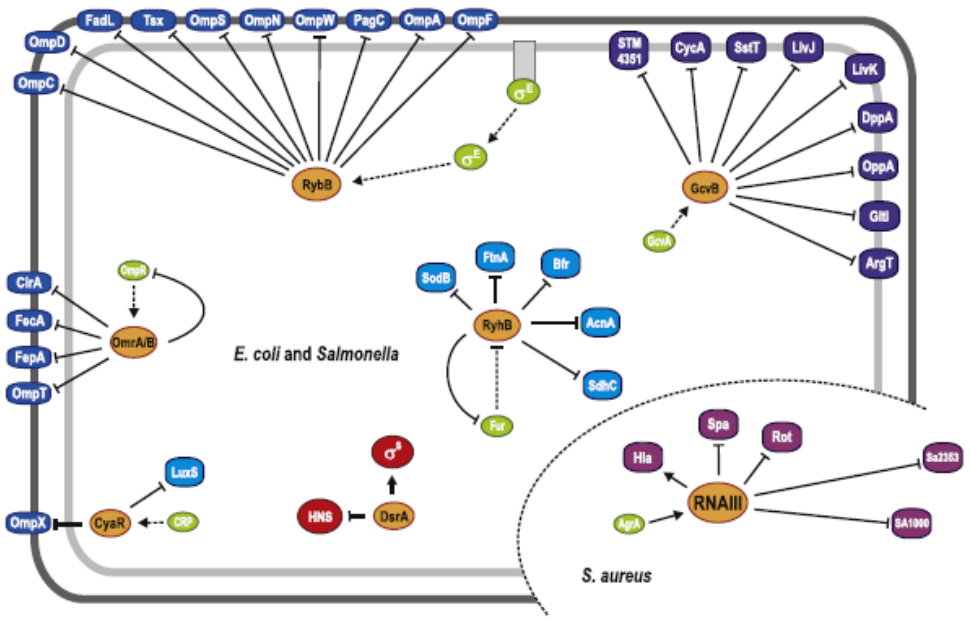

Fig. 6. Different targets of sRNAs in *E. coli, Salmonella* and *S. aureus*. sRNAs are shown in orange and their regulators are represented in green. In light, dark blue and red are represented the targets of the sRNAs in *E. coli* and *Salmonella* and in purple the targets in *S. aureus.* Adapted from (Papenfort and Vogel, 2009).

given stress condition, this biological particularity means that a single sRNA can globally modulate a particular physiological response, in much the same manner as a transcription factor, but at the post-transcriptional level. For example, it is intriguing that a disproportionate number of trans-encoded sRNAs regulate outer membrane proteins (MicA, MicC, MicF, RybB, CyaR, OmrA and OmrB) or transporters (SgrS, RydC, GcvB).

RNases are the enzymes that intervene in the processing, degradation and quality control of all types of RNAs, including the sRNAs and targets duplex. A limited number of RNases can exert a determinant level of control acting as a global regulatory network, monitoring and adapting the RNA levels to the cell needs. Different enzymes are involved in the RNA degradation mechanisms.

There are other accessory enzymes that can also intervene in these processes like helicases, polymerases (PAP) and RNA binding proteins.

mRNA degradation in the Gram-negative bacteria

In prokaryotes there are three possible pathways by which the mRNA molecules are degraded (FIG. 7). The more common model for RNA decay in *E. coli* (the model organism) usually begins with an endonucleolytic cleavage at one or more internal sites on the RNA molecule by the action of an endoribonuclease (either by RNase E, RNase G or by RNase III) (Arraiano et al., 2010; Carpousis et al., 2009). After the endonucleolytic cleavages, the transcripts are available for a direct exoribonucleolytic digestion to oligo- and mononucleotides by exoribonucleases such as RNase II, RNase R and/or PNPase (Coburn and Mackie, 1998). An alternative path for the RNA degradation relies on the endoribonuclease RNase E cleavage followed by polyadenylation by the action of the Poly(A) polymerase (PAP I). The addition of polyA tails to the 3´end of the mRNA molecules will "help" the activity of exoribonucleases, since these residues are the preferred substrate of some exoribonucleases. Furthermore, polyadenylation facilitates decay by providing a single-stranded platform for the 3'-exoribonucleases (Coburn and Mackie, 1998). The later mechanism described is of particular importance in the absence of endonucleolytic cleavages. In order to proceed with the degradation of mRNA molecules, polyadenylation is of extreme relevance for the removal of secondary structures. In this case mRNA decay can be achieved by successive cycles of polyadenylation followed by exoribonucleolytic cleavage (Regnier and Arraiano, 2000). The final step in the degradation pathway is the degradation of oligoribonucleotides by a oligoribonuclease of the short mRNA resulting from previews steps (Ghosh and Deutscher, 1999).

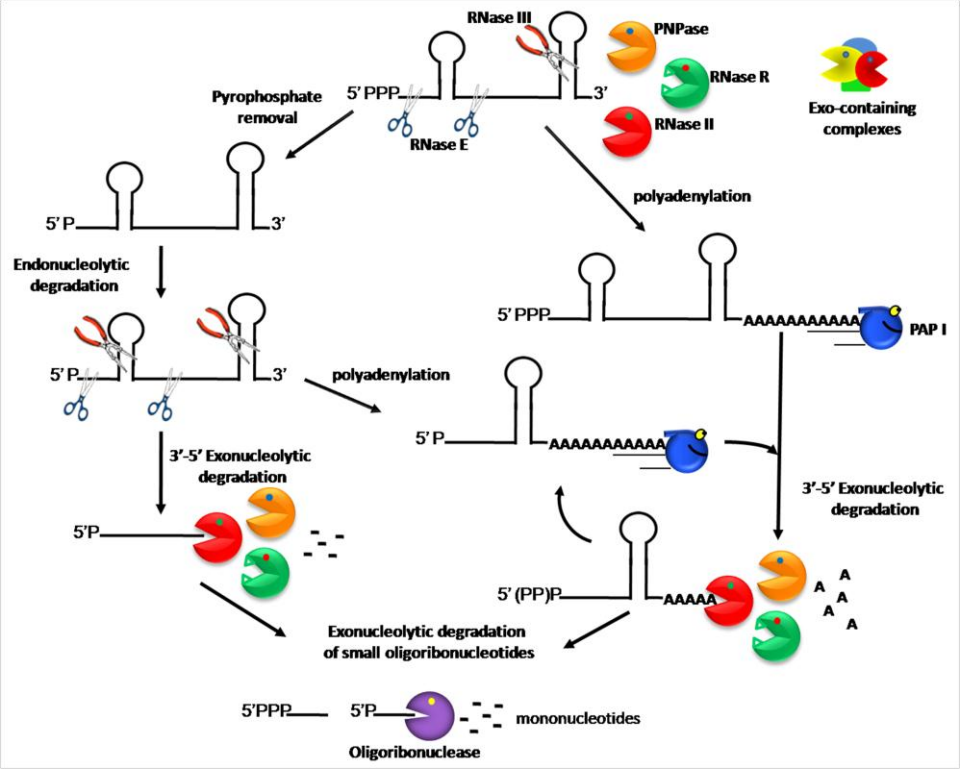

Fig. 7. Mechanisms of mRNA degradation pathway in *Escherichia coli*. The decay of the majority of transcripts starts with an endoribonucleolytic cleavage. After endoribonucleolytic cleavages, the linear transcripts are rapidly degraded by the 3′–5′ degradative exoribonucleases. The small oligoribonucleotides (two to five nucleotides) released by exoribonucleases are finally degraded to mononucleotides by oligoribonuclease Adapted from (Arraiano et al., 2010)

mRNA degradation in the Gram-positive bacteria

In the gram-positive model bacteria *B. subtilis,* RNase E is not present to start the mRNA decay pathway. RNase J1 seems to take over this function (FIG. 8) (Arraiano et al., 2010). Not so long ago, the exoribonucleolytic activity was believed to be just in the 3′to 5′direction. However, very recently this dogma was broken, since it was observed that, there is a 5'to 3' exoribonucleolytic activity in the maturation of 16S ribosomal RNA (Mathy et al., 2007). RNase J1 has been shown to have both endo and 5′–3′ exo activities and to have a major role in

mRNA turnover. It can be associated with RNase J2 or in a single unit form. For the initiation of endonuclease cleavage, RNase J1 either binds to the 5′ end or

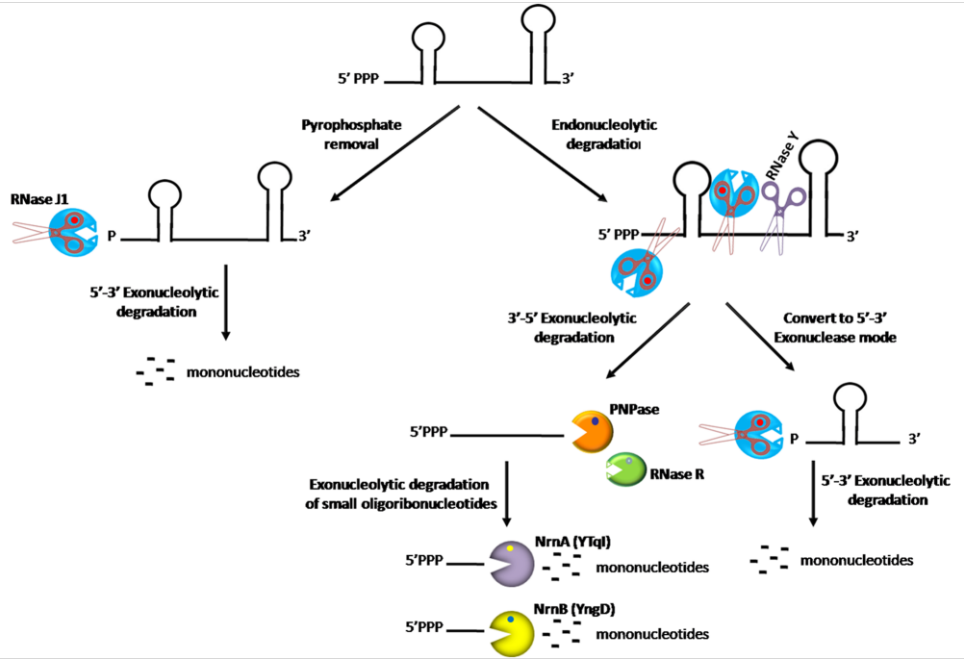

Fig. 8. Mechanisms of mRNA degradation pathway in *Bacillus subtilis*. RNAs can be degraded from the 5′ end or first, they can be endonucleolytically cleaved. The products from this endoribonucleolytic cleavage can then be degraded by the 3′–5′ exoribonucleases. The small oligoribonucleotides released by the 3′–5′ exoribonucleases are finally degraded to mononucleotides. Adapted from (Arraiano et al., 2010).

directly to the internal site of the mRNA. The upstream product is rapidly degraded by the 3′–5′ exonuclease activity of PNPase. Furthermore, the downstream RNA fragment with the 5′-monophosphate end can be a target of new RNase J1 endonuclease cleavage or processive 5′–3′ exonucleolytic decay from the 5′ end (Bechhofer, 2009). It was also shown that RNase J1 requires a single-stranded 5′ end with AU-rich regions to allow the exoribonucleolytic activity (Mathy et al., 2007). Another endonuclease sensitive to the 5′ end phosphorylation state of the substrate was recently discovered. RNase Y is involved in the initiation of turnover of *B. subtilis* S-adenosylmethionine-

dependent riboswitches (Shahbabian et al., 2009), which controls the expression of 11 transcriptional units (Henkin, 2008; Winkler and Breaker, 2005). The enzyme has a major function in the initiation of mRNA degradation in this organism, affecting mRNA stability >30% in an RNase J1/J2 double-mutant strain.

RNases are mostly responsible for the post-transcriptional control of RNA in bacteria. They are in charge not just of the mRNA degradation but are also involved in the quality control of the mRNA in the cells and processing of specific RNA molecules during their maturation. In many cases, these enzymes are also related with virulence. In the fourth part of this dissertation, we will study the role of *Streptococcus pneumoniae* RNase R, the unique hydrolytic ribonuclease described until now in this human pathogen.

RNase R and the post-transcriptional regulation

RNase R encoded by the *rnr* gene (previously *vacB*) is a 3′–5′ hydrolytic exoribonuclease from the RNase II family of exoribonucleases (Cheng and Deutscher, 2002; Vincent and Deutscher, 2006). In *E. coli, t*he *rnr* gene is in an operon and its transcription is driven from a putative σ^{70} promoter upstream of *nsrR* (Cairrao et al., 2003; Cheng et al., 1998). RNase R is a processive and sequence-independent enzyme, with a wide impact on RNA metabolism (Cairrao et al., 2003; Cheng and Deutscher, 2005; Andrade et al., 2006; Andrade et al., 2009; Purusharth et al., 2007). It is unique among the RNA-degradative exonucleases present in *E. coli* as it can easily degrade highly structured RNAs (Awano et al., 2010; Cheng and Deutscher, 2002; Cheng and Deutscher, 2003). This enzyme was seen to be involved in the control of gene expression (Andrade et al., 2006). Curiously, RNase R was shown to degrade the *ompA* transcript in a growth-phase-specific manner. This finding revealed a role for RNase R in the control of

gene expression that could not be replaced by any of the other exoribonucleases. The activity of RNase R is modulated according to the growth conditions of the cell and responds to environmental stimuli. RNase R levels are increased under several stresses, namely in cold shock, and the stationary phase of growth (Andrade et al., 2006; Cairrao et al., 2003). This protein is not essential for growth at optimal temperature; however, it is important for growth and viability at low temperatures (Charpentier et al., 2008). RNase R-like enzymes are widespread in most sequenced genomes. Even though most knowledge on this protein came from the work in *E. coli*, RNase R in other bacteria has been identified. Remarkably, RNase R has also been implicated in the establishment of virulence in a growing number of pathogens being involved in the modulation of the expression of virulence in a number of different pathogenic organisms (Cheng et al., 1998; Erova et al., 2008; Tobe et al., 1992; Tsao et al., 2009). In *Streptococcus pneumoniae*, there is a unique homologue of the RNase II family of enzymes that was shown to be an RNase R-like protein (Domingues et al., 2009). Proteins isolated from virulent and non-virulent *S. pneumonia* strains are different with respect to their activity and RNA affinity (Domingues et al., 2009). In the gram-positive model oganisms *B. subtilis,* RNase R was suggested not to play a critical role in RNA degradation; however, it may play a role in mRNA turnover when polyadenylation at the 3' end occurs (Oussenko et al., 2005). Moreover, *B. subtilis* RNase R was shown to be important for the quality control of tRNAs (Campos-Guillen et al., 2010). Overall, RNase R-deficient bacteria have been shown to be less virulent than the wild-type parental strains. However, how this is achieved is still not completely clear. This is probably related to critical RNA degradation pathways. The fact that RNase R was found to be the key in the degradation of sRNAs, namely the virulence regulator SsrA/tmRNA, is probably linked to its role in pathogenesis. It has also been suggested that RNase R may control the export of proteins involved in virulence mechanisms. Altogether, the available

data suggest that bacterial RNase R may be attractive as a potential therapeutic agent, but clearly more studies are required.

Aim of this Dissertation

In this Dissertation we wanted to focus on both transcriptional and post-transcriptional regulation of gene expression.

In a first part we have looked for possible regulators of *bolA.* We searched bioinformatically for putative candidates and H-NS was a possible regulator of *bolA*. By mRNA levels studies and different protein-DNA interaction techniques, we investigated if H-NS was regulating *bolA* and we have characterized its mode of action.

In a second part we aimed at the study of *bolA* gene as a transcription factor. It has been shown before that *bolA* was involved in the modulation of the mRNA levels of certain genes related with cell wall synthesis, cell wall structure and cell division. We have analyzed the impact of *bolA* overexpression and its absence in the *mreB* gene that codes for MreB protein that forms the bacterial "cytoskeleton".

We were also interested in studying the possible role of BolA protein in the modulation of non-coding RNAs, namely those transcribed by sigma E. In this part of the work we have studied how a transcription factor can have a role in post-transcriptional regulation by sRNAs.

Continuing our study on post-transcriptional control we have investigated the expression of a ribonuclease involved in RNA degradation in the pathogenic bacterium *Streptococcus pneumoniae.* We constructed an RNase R mutant (the unique hydrolytic exoribonuclease described in this organism) and characterized it regarding its expression, regulation and possible targets in this bacterium.

In summary, the theme of this thesis was focused on BolA and RNase R, two proteins that respectively affect transcription and post-transcriptional mechanisms in the cell. Every step on the path to understanding how gene expression is regulated is of major importance in any organism. The pleiotropic effects of *bolA* in cell morphology and cell division triggered our curiosity to study the regulation of this gene and understand how it affects such important mechanisms in the cell. Furthermore, deciphering the role of the until know unique RNase II family of enzymes in *Streptococcus pneumoniae*, the RNase R, was also of interest due to the involvement of this important post-transcriptional regulator in mRNA decay and virulence factors expression in this bacterium.

References

Aiba, H. 2007. Mechanism of RNA silencing by Hfq-binding small RNAs. *Curr Opin Microbiol*. 10:134-139.

Aldea, M., T. Garrido, C. Hernandez-Chico, M. Vicente, and S.R. Kushner. 1989. Induction of a growth-phase-dependent promoter triggers transcription of bolA, an Escherichia coli morphogene. *EMBO J*. 8:3923-3931.

Aldea, M., T. Garrido, J. Pla, and M. Vicente. 1990. Division genes in Escherichia coli are expressed coordinately to cell septum requirements by gearbox promoters. *EMBO J*. 9:3787-3794.

Aldea, M., C. Hernandez-Chico, A.G. de la Campa, S.R. Kushner, and M. Vicente. 1988. Identification, cloning, and expression of bolA, an ftsZ-dependent morphogene of Escherichia coli. *J Bacteriol*. 170:5169-5176.

Andrade, J.M., F. Cairrao, and C.M. Arraiano. 2006. RNase R affects gene expression in stationary phase: regulation of ompA. *Mol Microbiol*. 60:219-228.

Andrade, J.M., V. Pobre, I.J. Silva, S. Domingues, and C.M. Arraiano. 2009. The role of 3'-5' exoribonucleases in RNA degradation. *Prog Mol Biol Transl Sci*. 85:187-229.

Arraiano, C.M., J.M. Andrade, S. Domingues, I.B. Guinote, M. Malecki, R.G. Matos, R.N. Moreira, V. Pobre, F.P. Reis, M. Saramago, I.J. Silva, and S.C. Viegas. 2010. The critical role of RNA processing and degradation in the control of gene expression. *FEMS Microbiol Rev*. 34:883-923.

Awano, N., V. Rajagopal, M. Arbing, S. Patel, J. Hunt, M. Inouye, and S. Phadtare. 2010. Escherichia coli RNase R has dual activities, helicase and RNase. *J Bacteriol*. 192:1344-1352.

Bechhofer, D.H. 2009. Messenger RNA decay and maturation in Bacillus subtilis. *Prog Mol Biol Transl Sci*. 85:231-273.

Browning, D.F., and S.J. Busby. 2004. The regulation of bacterial transcription initiation. *Nat Rev Microbiol*. 2:57-65.

Cairrao, F., A. Cruz, H. Mori, and C.M. Arraiano. 2003. Cold shock induction of RNase R and its role in the maturation of the quality control mediator SsrA/tmRNA. *Mol Microbiol*. 50:1349-1360.

Campos-Guillen, J., J.L. Arvizu-Gomez, G.H. Jones, and G. Olmedo-Alvarez. 2010. Characterization of tRNA(Cys) processing in a conditional Bacillus subtilis CCase mutant reveals the participation of RNase R in its quality control. *Microbiology*. 156:2102-2111.

Carballido-Lopez, R. 2006. The bacterial actin-like cytoskeleton. *Microbiol Mol Biol Rev*. 70:888-909.

Carpousis, A.J., B.F. Luisi, and K.J. McDowall. 2009. Endonucleolytic initiation of mRNA decay in Escherichia coli. *Prog Mol Biol Transl Sci*. 85:91-135.

Charpentier, X., S.P. Faucher, S. Kalachikov, and H.A. Shuman. 2008. Loss of RNase R induces competence development in Legionella pneumophila. *J Bacteriol*. 190:8126-8136.

Cheng, Z.F., and M.P. Deutscher. 2002. Purification and characterization of the Escherichia coli exoribonuclease RNase R. Comparison with RNase II. *J Biol Chem*. 277:21624-21629.

Cheng, Z.F., and M.P. Deutscher. 2003. Quality control of ribosomal RNA mediated by polynucleotide phosphorylase and RNase R. *Proc Natl Acad Sci U S A*. 100:6388-6393.

Cheng, Z.F., and M.P. Deutscher. 2005. An important role for RNase R in mRNA decay. *Mol Cell*. 17:313-318.

Cheng, Z.F., Y. Zuo, Z. Li, K.E. Rudd, and M.P. Deutscher. 1998. The vacB gene required for virulence in Shigella flexneri and Escherichia coli encodes the exoribonuclease RNase R. *J Biol Chem*. 273:14077-14080.

Coburn, G.A., and G.A. Mackie. 1998. Reconstitution of the degradation of the mRNA for ribosomal protein S20 with purified enzymes. *J Mol Biol*. 279:1061-1074.

deHaseth, P.L., M.L. Zupancic, and M.T. Record, Jr. 1998. RNA polymerase-promoter interactions: the comings and goings of RNA polymerase. *J Bacteriol*. 180:3019-3025.

Domingues, S., R.G. Matos, F.P. Reis, A.M. Fialho, A. Barbas, and C.M. Arraiano. 2009. Biochemical characterization of the RNase II family of

exoribonucleases from the human pathogens Salmonella typhimurium and Streptococcus pneumoniae. *Biochemistry*. 48:11848-11857.

Dove, S.L., S.A. Darst, and A. Hochschild. 2003. Region 4 of sigma as a target for transcription regulation. *Mol Microbiol*. 48:863-874.

Ebright, R.H. 2000. RNA polymerase: structural similarities between bacterial RNA polymerase and eukaryotic RNA polymerase II. *J Mol Biol*. 304:687-698.

Erova, T.E., V.G. Kosykh, A.A. Fadl, J. Sha, A.J. Horneman, and A.K. Chopra. 2008. Cold shock exoribonuclease R (VacB) is involved in Aeromonas hydrophila pathogenesis. *J Bacteriol*. 190:3467-3474.

Fozo, E.M., M.R. Hemm, and G. Storz. 2008. Small toxic proteins and the antisense RNAs that repress them. *Microbiol Mol Biol Rev*. 72:579-589, Table of Contents.

Freire, P., J.D. Amaral, J.M. Santos, and C.M. Arraiano. 2006a. Adaptation to carbon starvation: RNase III ensures normal expression levels of bolAp1 mRNA and sigma(S). *Biochimie*. 88:341-346.

Freire, P., R.N. Moreira, and C.M. Arraiano. 2009. BolA inhibits cell elongation and regulates MreB expression levels. *J Mol Biol*. 385:1345-1351.

Freire, P., H.L. Vieira, A.R. Furtado, M.A. de Pedro, and C.M. Arraiano. 2006b. Effect of the morphogene bolA on the permeability of the Escherichia coli outer membrane. *FEMS Microbiol Lett*. 260:106-111.

Ghosh, S., and M.P. Deutscher. 1999. Oligoribonuclease is an essential component of the mRNA decay pathway. *Proc Natl Acad Sci U S A*. 96:4372-4377.

Gottesman, S., and G. Storz. 2010. Bacterial Small RNA Regulators: Versatile Roles and Rapidly Evolving Variations. *Cold Spring Harb Perspect Biol*.

Grainger, D.C., D. Hurd, M.D. Goldberg, and S.J. Busby. 2006. Association of nucleoid proteins with coding and non-coding segments of the Escherichia coli genome. *Nucleic Acids Res*. 34:4642-4652.

Gross, C.A., C. Chan, A. Dombroski, T. Gruber, M. Sharp, J. Tupy, and B. Young. 1998. The functional and regulatory roles of sigma factors in transcription. *Cold Spring Harb Symp Quant Biol*. 63:141-155.

Guinote, I.B., R.G. Matos, P. Freire, and C.M. Arraiano. 2011. BolA affects cell growth, and binds to the promoters of penicillin-binding proteins 5 and 6 and regulates their expression. *J Microbiol Biotechnol*. 21:243-251.

Helmann, J.D., and M.J. Chamberlin. 1988. Structure and function of bacterial sigma factors. *Annu Rev Biochem*. 57:839-872.

Henkin, T.M. 2008. Riboswitch RNAs: using RNA to sense cellular metabolism. *Genes Dev*. 22:3383-3390.

Ishihama, A. 1997. Adaptation of gene expression in stationary phase bacteria. *Curr Opin Genet Dev*. 7:582-588.

Ishihama, A. 2000. Functional modulation of Escherichia coli RNA polymerase. *Annu Rev Microbiol*. 54:499-518.

Jordi, B.J., and C.F. Higgins. 2000. The downstream regulatory element of the proU operon of Salmonella typhimurium inhibits open complex formation by RNA polymerase at a distance. *J Biol Chem*. 275:12123-12128.

Lange, R., and R. Hengge-Aronis. 1991. Growth phase-regulated expression of bolA and morphology of stationary-phase Escherichia coli cells are controlled by the novel sigma factor sigma S. *J Bacteriol*. 173:4474-4481.

Madan Babu, M., and S.A. Teichmann. 2003. Evolution of transcription factors and the gene regulatory network in Escherichia coli. *Nucleic Acids Res*. 31:1234-1244.

Maeda, H., N. Fujita, and A. Ishihama. 2000. Competition among seven Escherichia coli sigma subunits: relative binding affinities to the core RNA polymerase. *Nucleic Acids Res*. 28:3497-3503.

Martinez-Antonio, A., and J. Collado-Vides. 2003. Identifying global regulators in transcriptional regulatory networks in bacteria. *Curr Opin Microbiol*. 6:482-489.

Mathy, N., L. Benard, O. Pellegrini, R. Daou, T. Wen, and C. Condon. 2007. 5'-to-3' exoribonuclease activity in bacteria: role of RNase J1 in rRNA maturation and 5' stability of mRNA. *Cell*. 129:681-692.

McLeod, S.M., and R.C. Johnson. 2001. Control of transcription by nucleoid proteins. *Curr Opin Microbiol*. 4:152-159.

Muller-Hill, B. 1998. Some repressors of bacterial transcription. *Curr Opin Microbiol*. 1:145-151.

Murakami, K.S., S. Masuda, E.A. Campbell, O. Muzzin, and S.A. Darst. 2002. Structural basis of transcription initiation: an RNA polymerase holoenzyme-DNA complex. *Science*. 296:1285-1290.

Oshima, T., S. Ishikawa, K. Kurokawa, H. Aiba, and N. Ogasawara. 2006. Escherichia coli histone-like protein H-NS preferentially binds to horizontally acquired DNA in association with RNA polymerase. *DNA Res*. 13:141-153.

Oussenko, I.A., T. Abe, H. Ujiie, A. Muto, and D.H. Bechhofer. 2005. Participation of 3'-to-5' exoribonucleases in the turnover of Bacillus subtilis mRNA. *J Bacteriol*. 187:2758-2767.

Papenfort, K., and J. Vogel. 2009. Multiple target regulation by small noncoding RNAs rewires gene expression at the post-transcriptional level. *Res Microbiol*. 160:278-287.

Perez-Rueda, E., and J. Collado-Vides. 2000. The repertoire of DNA-binding transcriptional regulators in Escherichia coli K-12. *Nucleic Acids Res*. 28:1838-1847.

Petersen, C., L.B. Moller, and P. Valentin-Hansen. 2002. The cryptic adenine deaminase gene of Escherichia coli. Silencing by the nucleoid-associated DNA-binding protein, H-NS, and activation by insertion elements. *J Biol Chem*. 277:31373-31380.

Prevost, K., H. Salvail, G. Desnoyers, J.F. Jacques, E. Phaneuf, and E. Masse. 2007. The small RNA RyhB activates the translation of shiA mRNA encoding a permease of shikimate, a compound involved in siderophore synthesis. *Mol Microbiol*. 64:1260-1273.

Purusharth, R.I., B. Madhuri, and M.K. Ray. 2007. Exoribonuclease R in Pseudomonas syringae is essential for growth at low temperature and plays a novel role in the 3' end processing of 16 and 5 S ribosomal RNA. *J Biol Chem*. 282:16267-16277.

Regnier, P., and C.M. Arraiano. 2000. Degradation of mRNA in bacteria: emergence of ubiquitous features. *Bioessays*. 22:235-244.

Santos, J.M., D. Drider, P.E. Marujo, P. Lopez, and C.M. Arraiano. 1997. Determinant role of E. coli RNase III in the decay of both specific and heterologous mRNAs. *FEMS Microbiol Lett*. 157:31-38.

Santos, J.M., P. Freire, F.S. Mesquita, F. Mika, R. Hengge, and C.M. Arraiano. 2006. Poly(A)-polymerase I links transcription with mRNA degradation via sigmaS proteolysis. *Mol Microbiol*. 60:177-188.

Santos, J.M., P. Freire, M. Vicente, and C.M. Arraiano. 1999. The stationary-phase morphogene bolA from Escherichia coli is induced by stress during early stages of growth. *Mol Microbiol*. 32:789-798.

Santos, J.M., M. Lobo, A.P. Matos, M.A. De Pedro, and C.M. Arraiano. 2002. The gene bolA regulates dacA (PBP5), dacC (PBP6) and ampC (AmpC), promoting normal morphology in Escherichia coli. *Mol Microbiol*. 45:1729-1740.

Schnetz, K. 1995. Silencing of Escherichia coli bgl promoter by flanking sequence elements. *EMBO J*. 14:2545-2550.

Shahbabian, K., A. Jamalli, L. Zig, and H. Putzer. 2009. RNase Y, a novel endoribonuclease, initiates riboswitch turnover in Bacillus subtilis. *EMBO J*. 28:3523-3533.

Tobe, T., C. Sasakawa, N. Okada, Y. Honma, and M. Yoshikawa. 1992. vacB, a novel chromosomal gene required for expression of virulence genes on the large plasmid of Shigella flexneri. *J Bacteriol*. 174:6359-6367.

Tsao, M.Y., T.L. Lin, P.F. Hsieh, and J.T. Wang. 2009. The 3'-to-5' exoribonuclease (encoded by HP1248) of Helicobacter pylori regulates motility and apoptosis-inducing genes. *J Bacteriol*. 191:2691-2702.

Valentin-Hansen, P., M. Eriksen, and C. Udesen. 2004. The bacterial Sm-like protein Hfq: a key player in RNA transactions. *Mol Microbiol*. 51:1525-1533.

Vieira, H.L., P. Freire, and C.M. Arraiano. 2004. Effect of Escherichia coli morphogene bolA on biofilms. *Appl Environ Microbiol*. 70:5682-5684.

Vincent, H.A., and M.P. Deutscher. 2006. Substrate recognition and catalysis by the exoribonuclease RNase R. *J Biol Chem*. 281:29769-29775.

Waters, L.S., and G. Storz. 2009. Regulatory RNAs in bacteria. *Cell*. 136:615-628.

Winkler, W.C., and R.R. Breaker. 2005. Regulation of bacterial gene expression by riboswitches. *Annu Rev Microbiol*. 59:487-517.

Wosten, M.M. 1998. Eubacterial sigma-factors. *FEMS Microbiol Rev*. 22:127-150.

Chapter 2

A new target for an old regulator: H-NS represses transcription of *bolA* morphogene by direct binding to both promoters

This chapter contains data published in:
Moreira, R.N., C. Dressaire, S. Domingues, and C.M. Arraiano. 2011. A new target for an old regulator: H-NS represses transcription of *bolA* morphogene by direct binding to both promoters. **Biochem Biophys Res Commun. 411:50-55**

Abstract

The Escherichia coli *bolA* morphogene is very important in adaptation to stationary phase and stress response mechanisms. Genes of this family are widespread in gram negative bacteria and in eukaryotes. The expression of this gene is tightly regulated at transcriptional and post-transcriptional levels and its overexpression is known to induce round cellular morphology. The results presented in this report demonstrate that the H-NS protein, a pleiotropic regulator of gene expression, is a new transcriptional modulator of the *bolA* gene. In this work we show that *in vivo* the levels of *bolA* are down-regulated by H-NS and *in vitro* this global regulator interacts directly with the *bolA* promoter region. Moreover, DNaseI footprinting experiments mapped the interaction regions of H-NS and *bolA* and revealed that this global regulator binds not only one but both *bolA* promoters. We provide a new insight into the *bolA* regulation network demonstrating that H-NS represses the transcription of this important gene.

Introduction

The *E. coli bolA* gene is induced at the onset of stationary phase and in response to many forms of stress (Santos et al., 1999), The overexpression of *bolA* leads to substantial changes in the cell and the bacterial bacilli transform into spheres (Aldea et al., 1989; Aldea et al., 1988; Santos et al., 1999). The fact that BolA affects the expression of numerous genes highlights its importance, and previous reports show that *bolA* can act as a transcription factor. For instance, BolA has been demonstrated to specifically interact with the *mreB* promoter, repressing its transcription (Freire et al., 2009). This leads to a reduction in MreB protein levels and consequently to an abnormal MreB polymerization. BolA was also shown to directly regulate the transcript levels of the important D,D-carboxypeptidases PBP5 and PBP6, and to modulate the expression levels of the β-lactamase AmpC (Aldea et al., 1988; Santos et al., 2002). Furthermore, *bolA* is involved in biofilm development and promotes changes in the outer membrane that affect permeability and resistance to antibiotics such as vancomycin (Freire et al., 2006b; Vieira et al., 2004). It is not surprising that the expression of a gene involved in the control of several cellular processes is tightly regulated at transcriptional and post-transcriptional levels. In optimal growth conditions, *bolA* is under the control of a weak σ^{70}-dependent constitutive promoter, bolAp2. During stress and stationary phase it is mostly transcribed from the strong gearbox promoter, bolAp1, induced by the sigma factor σ^{s} (Aldea et al., 1989; Nguyen and Burgess, 1997). As a σ^{S}-regulated gene, *bolA* expression is sensitive to ppGpp (Gentry et al., 1993) and cAMP (Lange and Hengge-Aronis, 1991) intracellular levels. *bolA* was also shown to be repressed by the direct binding of OmpR in its phosphorylated form (Yamamoto et al., 2000). Ribonuclease III (RNase III) and polyA polymerase (PAPI) are involved in post-transcriptional

control of *bolA* expression (Arraiano et al., 2010; Freire et al., 2006a; Santos et al., 2006).

The histone-like (or heat-stable) protein H-NS was shown to affect some σ^S-dependent genes (Barth et al., 1995). This 15kDa nucleoid-associated protein is abundant in bacterial cells and is often compared to eukaryotic histones because of its high affinity for DNA. It binds preferentially to curved AT-rich regions that are found in certain promoter regions (Dorman, 2004).

In this work we show that H-NS down-regulates *bolA* levels. We demonstrate that this regulation is mediated by a specific binding of H-NS to the *bolA* promoter region, involving both promoters. The interaction region of H-NS with *bolA* promoters was mapped and the implications of *bolA* regulation by H-NS are discussed.

Results & Discussion

Effect of H-NS in bolA expression

In optimal growth conditions during exponential phase *bolA* is regulated by σ^{70} and only relatively low mRNA levels are detected in the cell. However in stationary phase, *bolA* expression is under the control of σ^{s} and a 5-fold increase of the transcript level is observed (Santos et al., 1999). Under stress conditions the *bolA* levels can increase further (Santos et al., 1999). Since H-NS is a global regulator shown to affect the expression of several genes that respond to stress and are regulated by σ^{s}, we wanted to test whether H-NS could also be involved in the control of *bolA* expression. Taking into account that *bolA* is growth-phase regulated; the influence of H-NS on its expression was assayed during different phases of bacterial growth. Three points were analyzed (OD_{600} of 0.4, 1.2 and 2.5) corresponding to exponential, late exponential and stationary phase. For each optical density, samples were taken and total RNA was extracted from a wt cell culture and the isogenic Δ*hns* strain. *bolA* mRNA levels were then estimated by RT-PCR using a pair of specific primers (Figure 1). In agreement with the previous results for the wild type strain, *bolA* levels are low during exponential phase and reach a maximum during stationary phase. In the absence of H-NS, the *bolA* levels in late exponential phase are significantly higher than in the wt and increase ~2.4 fold. In the *hns* strain there is also an increase of *bolA* in stationary phase but the difference is quite lower. H-NS is probably repressing the expression of *bolA* during late exponential growth similarly to what happens when it regulates the *hchA* gene (Mujacic and Baneyx, 2006). On the other hand, in stationary phase competes with the higher σ^{S} levels in the cell, and that is probably why it cannot exert the same level of repression.

Taken together, these results indicate that H-NS downregulates *bolA* expression, supporting our hypothesis that H-NS could be a transcriptional repressor of *bolA* expression. Moreover, these results provide another evidence for the key function of H-NS as a selective silencer of genes that rapidly respond to environmental changes (Barth et al., 1995; Lang et al., 2007; White-Ziegler and Davis, 2009).

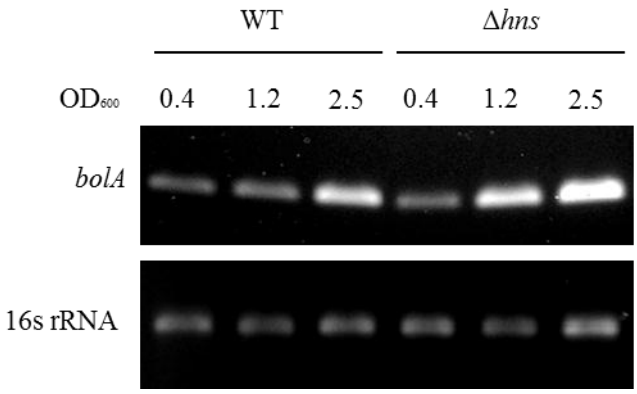

Fig. 1. Down-regulation of the *bolA* transcript level by H-NS.
RT-PCR amplification of *bolA* transcript from total RNA extracted in different growth phases: *E. coli* exponential (OD_{600} 0.4), late exponential (OD_{600} 1.2) and stationary phase (OD_{600} 2.5) (upper image). Control experiments performed with 16s rRNA specific primers (image on the bottom) indicated that there were no significant differences in the amounts of RNA in each sample.

H-NS binds specifically to bolA promoter(s)

The RT-PCR results indicate that H-NS is involved in the modulation of *bolA* mRNA levels. However the nature of this regulation remains unknown. H-NS is known to be able to modulate gene expression in at least two different ways: by directly binding to specific targets or indirectly through the down-regulation of the σ^S transcript (Barth et al., 1995). Thus we tested if H-NS could be acting directly over bolA as a transcriptional regulator. For this purpose, the *E. coli* H-NS protein was purified near homogeneity (Figure S1) and the pure protein was used

in gel mobility shift assays with the *bolA* genomic region. Four different substrates were used in order to discriminate the ability of H-NS to bind the *bolA* upstream region (Figure 2A). As a protein that binds DNA with high affinity, H-NS was able to retard the mobility of all the DNA fragments tested, generating retardation bands that correspond to DNA-protein complexes (Figure 2B). However, some relevant differences were observed among the substrates tested. The substrate 1, comprising only bolAp2 and bolAp1 is clearly the preferred H-NS substrate. For this substrate DNA-Protein complexes could be observed with only 0.3μM of H-NS, while at least a 2-fold excess was needed for the formation of DNA-protein complexes with any of the other substrates. In addition, when using substrate 1 almost all DNA was bound with only 0.9μM of H-NS, whereas the amount of protein needed to completely bind the substrate 2 (bolAp2+bolAp1+ORF) raised to 1.2μM. This amount of H-NS was not even sufficient to completely bind substrate 3 (missing bolAp2), and at this protein concentration free DNA was still detected. At higher H-NS concentrations, a retarded band of higher molecular mass could be detected. The appearance of this band was concomitant with the disappearance of the complex of lower mass. The higher band probably corresponds to the binding of more than one molecule per substrate. H-NS is indeed known to form higher order structure complexes with its targets (Fang and Rimsky, 2008). With substrate 1, at 1.2μM almost all DNA molecules seem to be bound by more than one protein molecule. When using substrate 2 with the same H-NS concentration, this higher order complex is almost absent, indicating that the majority of DNA is still bound by only one H-NS molecule. The substrate missing bolAp2 presents an intermediate situation since both protein complexes are equally detected. These experiments show that *in vitro* the presence of the whole *bolA* coding region (substrate 2) or the deletion of bolAp2 (substrate 3) seems to affect the efficiency of the H-NS binding to *bolA*.

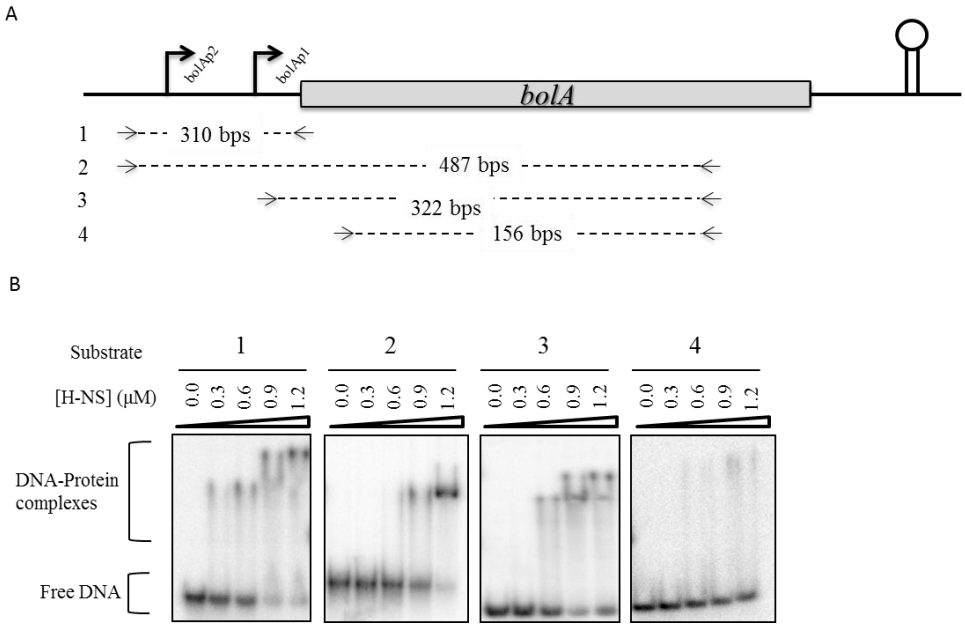

Fig. 2. DNA-Protein interactions of *bolA* promoters and H-NS.
(A) Schematic representation of *bolA* genomic region. The different substrates used in the electro-mobility shift assays (EMSA) are represented. (B) Representative EMSA of H-NS with 1nM of the indicated substrates above the respective image. A control reaction without protein ([H-NS] = 0.0 μM) was performed in all experiments. Binding reactions using an increasing concentration of H-NS (indicated at the top of each lane) were resolved in a 5 % non-denaturing polyacrylamide gel. Free DNA and DNA-protein complexes are indicated.

Finally, H-NS was also able to bind to the substrate which comprises only the *bolA* coding region (substrate 4), although with a significantly lower affinity. Together, these results suggest that H-NS binds preferentially the *bolA* promoter region (with both promoters). H-NS is not only sequence but also structure sensitive (Lang et al., 2007). Despite bolAp1 and bolAp2 being present in substrate 2, the additional presence of the *bolA* ORF may change the conformation

of the promoter region (see below), thus affecting H-NS binding. This likely explains the partial loss of H-NS binding affinity for the longer substrate.

These experiments clearly show that the efficiency of H-NS binding is affected by both *bolA* promoters. Together with the in vivo data these results provide strong evidence that H-NS represses *bolA* transcription through a direct interaction with the entire *bolA* promoter region. Our results indicate that the reported co-immunoprecitation of *bolA* with H-NS (Dorman, 2004) was probably due to a direct interaction with this *bolA* region.

DNA curvature of bolA promoter region

DNA Curvature Analysis bioinformatics tool was used to calculate the bending region(s) to evaluate if 3D structure of the *bolA* promoters could influence H-NS binding. The double helix of a DNA fragment containing both *bolA* promoters displayed an accentuated curvature, possibly favouring the interaction with H-NS (Figure S2). However, when a DNA fragment lacking bolAp2 was analysed (such as substrate 3), the curvature is close to null. Hence, the DNA curvature seems to be directly dependent on the bolAp2 region. Since the DNA structure is an important factor for the proper H-NS binding specificity, this could be one reason for the variations observed above (in the gel retardation assays).

Mapping of H-NS interaction sites

To pursue our studies and clearly identify the region(s) of interaction between H-NS and the *bolA* promoters we have performed DNaseI foot-printing assays to map the H-NS binding sites to the *bolA* promoters. We used a DNA fragment containing both bolAp2 and bolAp1 (substrate 1-Fig 2) and protection zones were identified (Figure 3). The interaction regions were evenly distributed through the entire region analysed, which demonstrates that H-NS can bind to

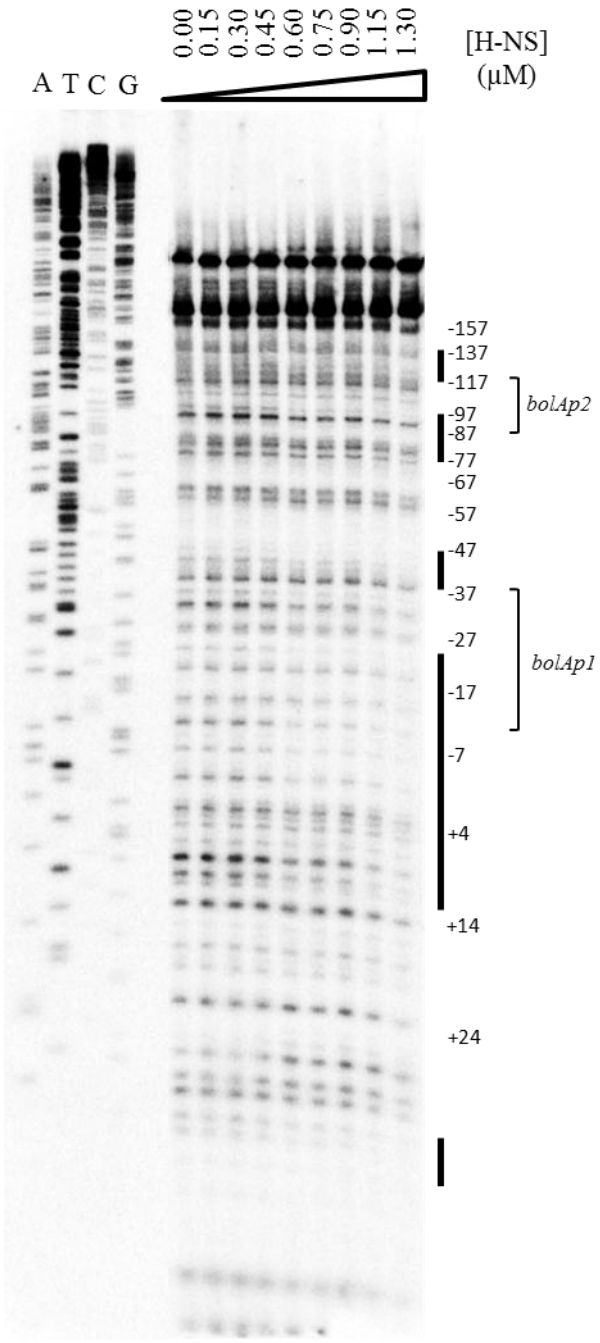

Fig. 3. Mapping of H-NS binding sites on *bolA* promoters by DNaseI foot-printing.
Electrophoretic separation of a fragment with *bolAp2* and *bolAp1* after H-NS binding followed by DNaseI digestion. The different lanes correspond to different H-NS concentrations, as indicated on top. The lanes labeled with A, T, C and G represent the M13 sequencing reaction. The sites that are protected by H-NS are indicated with black lines. The numbers indicate nucleotide positions with respect to the *bolAp1* transcription start site.

several sites covering both *bolA* promoters. Sequence analysis demonstrated that the largest protection site was detected in the gearbox promoter bolAp1 (Figure 4). A narrower protection zone was found upstream of the -35 box of this promoter. Two other main interaction regions were mapped around bolAp2. The last protection zone corresponds only to a 3 bps sequence and it may not be significant. Even though H-NS was considered a non-sequence specific binding

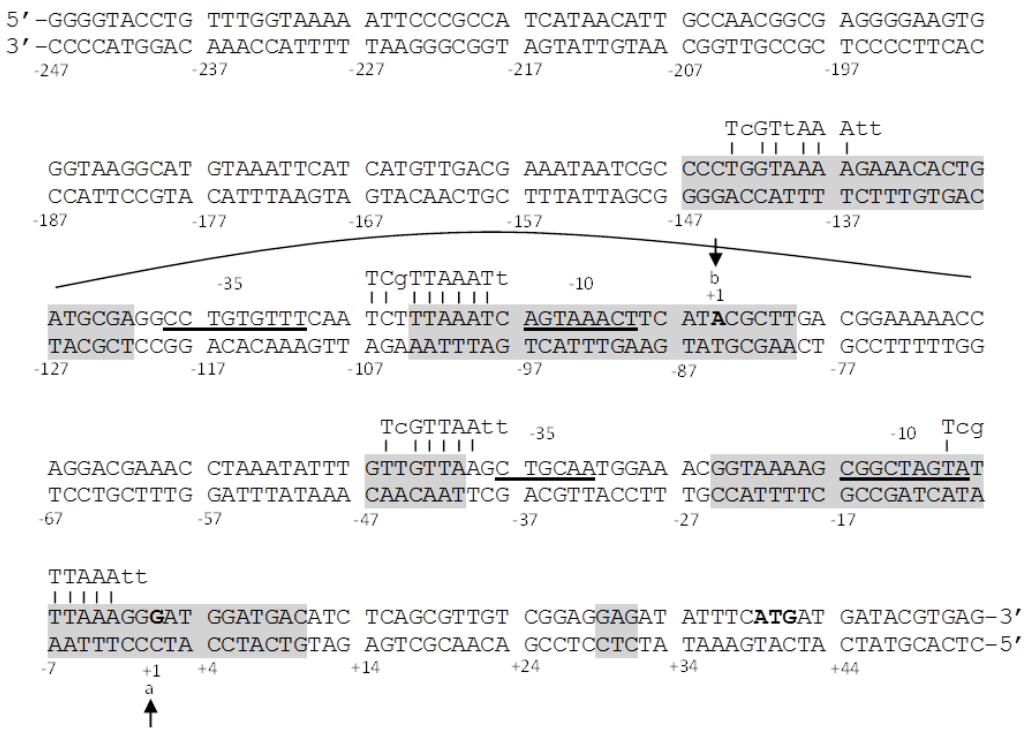

Fig. 4. Schematic representation of *bolA* promoter region.
The numbers indicate nucleotide positions with respect to the *bolAp1* transcription start site. The sites revealed by H-NS-mediated DNaseI protection are highlighted in gray and the -35 and -10 regions of the promoters are underlined. The transcription start sites (a and b), and the initiation codon are in bold. The H-NS-binding consensus sequence is indicated above the DNA duplex, and the vertical bars indicate the base match between consensus and promoter sequence. The region of DNA predicted to have significant curvature is indicated by a curved line immediately above the sequence.

protein, recent studies defined that this global regulatory protein interacts with AT-rich regions commonly found in bacterial gene promoters (Lang et al., 2007). A consensus region, and a consensus structure (DNA curvature) for protein-DNA interaction has also been identified (Lang et al., 2007; Sette et al., 2009; Yamada et al., 1990). In these experiments, the regions of interaction were confirmed to be AT-rich, matching the characteristics of the high affinity H-NS interaction zones and, all the main interaction zones identified share a partial similarity with the 10 bp described consensus (TCGTTAAATT) (Lang et al., 2007) (see Figure 4). Altogether, our results support H-NS ability to bind simultaneously to several sites within the entire regulatory region of *bolA*, and form higher order structures originating a repressive nucleoprotein complex that modulates the activity of bolAp1 and bolAp2.

In this report we showed that the pleiotropic histone-like protein H-NS is a new transcription regulator of *bolA* and we have characterized its mode of action. We demonstrated that H-NS is directly repressing *bolA* expression by binding to different locations along its entire promoter regions. Four major interaction zones were identified encompassing both bolAp2 and bolAp1 promoters. Moreover, the binding sites are confined to a curved DNA region, acknowledged to be the H-NS preferred consensus structure.

BolA has been shown to be a pleiotropic protein that affects several cellular functions. It has been described as a transcription factor, as well as a morphogene (Aldea et al., 1988; Freire et al., 2009; Guinote, 2011). It was also shown to be important for cell survival (Freire et al., 2006b). In this context, a fine tuned regulation of this gene may be essential for the cell. This work adds a new regulator, H-NS, to the already complex network of BolA modulators. H-NS is known to be involved in flagella biosynthesis (Bertin et al., 1994). Additionally, in *E. coli*, bacterial motility influence biofilms architecture (Wood et al., 2006). We

have previously shown that *bolA* can induce biofilm formation (Vieira et al., 2004), therefore H-NS and BolA may be involved in the molecular mechanisms that control the link between motility and biofilm development.

Materials and Methods

Materials

Restriction enzymes, T4 DNA ligase, Pfu DNA polymerase and T4 Polynucleotide Kinase were purchased from Fermentas. DNaseI was purchased from Sigma. All the enzymes were used according to the supplier's instructions. Oligonucleotide primers used in this work are listed in Table I and were synthesized by STAB Vida, Portugal.

Table 1. Oligonucleotides used in this work

Oligonucleotides	Sequence
hnsNdeI	5'-GGAATTCCATATGAGCGAAGCACTTAAAATTCTG-3'
hnsBamHI	5'-CGGGATCCCGTTATTGCTTGATCAGGAAATCGTCGAGGG-3'
X2	5'-GTCACAATGTCCCAGCCG-3'
X7	5'-CGATGCTTCCTGCTCCAC-3'
16sF	5'-AGAGTTTGATCCTGGCTCAG-3'
16sR	5'-ACGGCTACCTTGTTACGACTT-3'
bolAFw	5'-GGGGTACCTGTTTGGTAAAAATTCCCG-3'
RNM012	5'-TCTATCCGCTCACGTATCAT-3'
RblrealT	5'-AGTTCCTCCGCTAAAGTACTG-3'
P2	5'-CTTGACGGAAAAACCAGGACG-3'
FblrealT	5'-AACCCGTATTCCTCGAAGTAG-3'

Bacterial Strains and Plasmids

The *E. coli* strains used were: DH5α (F' *fhuA2* Δ*(argF-lacZ)U169 phoA glnV44 Φ80* Δ*(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17a*) for cloning experiments; BL21(DE3) *(F- rB- mB- gal ompT (int::PlacUV5 T7 gen1 imm21 nin5)* for overexpression and purification of the H-NS protein; JW1225 (*Δhns::kan*^{r}) (Baba et al., 2006); MG1655; MG1693 (a spontaneous Thy- derivative of strain MG1655); and CMA92 (MG1655 *Δhns::kan*^{r}), this work. These strains were grown in Luria Broth medium (LB) at 37°C, supplemented with 100μg/ml ampicillin or 50μg/ml kanamycin, when required.

The *hns* coding sequence was amplified by PCR using *E. coli* MG1693 chromosomal DNA and the primers hnsNdeI and hnsBamHI. The amplified fragment was cut with NdeI and BamHI restriction enzymes and cloned into the pET-15b vector (Novagen) previously cleaved with the same enzymes. The resulting plasmid (pCDA1) encoding H-NS fused to an N-terminal His6-tag was used to transform E. coli BL21(DE3) resulting in CMA93 strain (BL21(DE3)+pCDA1).

The *hns* deletion mutant was obtained from the Keio collection (Baba et al., 2006). P1-mediated transduction to transfer the mutation to the MG1655 background (CMA92) was performed as previously described (Sambrook and Russell, 2001). All constructions were confirmed by DNA sequencing at STAB Vida, Portugal.

Expression and purification of H-NS

BL21(DE3) containing pCDA1 was grown overnight at 37°C, 120rpm in LB media supplemented with 100μg.ml^{-1} ampicillin. Fresh 250ml of LB was inoculated with the overnight culture to a final OD_{600} of 0.1 and the culture was incubated at 37°C, 180rpm. At OD_{600} ~ 0.5, the expression of *hns* was induced with

1mM IPTG for 2h in the same growing conditions. Cells were harvested by centrifugation and the pellets stored at −80°C. The cellular pellets were resuspended in 6ml of buffer A (20mM sodium phosphate, 0.5M NaCl, 20mM imidazole, pH7.4) supplemented with 0.1mM phenylmethylsulfonyl fluoride (PMSF). Cells were then disrupted using a French press at 9000psi and the crude extracted was treated with Benzonase (Sigma) to degrade the nucleic acids. After 30min incubation on ice, the suspension was centrifuged for 30min, at 48000xg, 4°C. The supernatant was collected and loaded into a HisTrap Chelating Sepharose 1ml column (GE Healthcare) equilibrated in buffer A using an AKTA HLPC system (GE Healthcare). Elution was performed using a gradient of buffer B (20mM sodium phosphate, 0.5M NaCl, 500mM Imidazole, pH7.4) from 0% to 100% in 20min. Collected fractions containing the pure protein were pooled together and buffer exchanged to Buffer C (20mM sodium phosphate, 0.5M NaCl, pH7,4) using a desalting 5ml column (GE Healthcare). Eluted proteins were then concentrated by centrifugation at 4°C with an Amicon Ultra Centrifugal Filters Devices (Millipore) with a mass cutoff of 10kDa. Protein concentration was determined by the Bradford quantification method and 50% (v/v) glycerol was added to the final fractions prior to storage at -20°C. More than 90% homogeneity as revealed by analyzing the purified protein in a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) stained with Coomassie blue.

RNA Extraction and RT-PCR

Total RNA was extracted by the Trizol (Ambion) according to the supplier instructions with some modifications. Briefly, an overnight CMA92 culture was diluted to a final OD_{600} of 0.1 and incubated at 37°C, 180rpm. Samples were collected at different points corresponding to the different phases of the bacterial growth curve (exponential – 0.4; late exponential – 1.2; and stationary

phase – 2.5). Each aliquot containing 20ml of bacterial cell culture was mixed with an equal volume of TM buffer (10mM Tris, 25mM NaN3, 500µg/ml chloramphenicol, 5mM MgCl2, pH7.2) and harvested by centrifugation. The cell pellet was resuspendend in 600µl of lysis buffer (10mM Tris, 5mM MgCl2, 300µg/ml lysozyme, pH7.2) followed by five cycles of freeze and thaw. The suspension was supplemented with 1% SDS and 0.33mM AcOH. 1ml of Trizol reagent (Ambion) was added and the suspension was vortexed 5min at room temperature, followed by a 10min centrifugation at 16000xg, 4°C. The aqueous phase was collected and mixed with 200µl of chloroform. The mixture was vortexed again for 15min at room temperature and centrifuged for 15min at 4°C. The aqueous phase was collected and total RNA was precipitated with isopropanol. After drying, the pellet was resuspended in H2O and the RNA concentration was measured with a spectrophotometer (NanoDrop 1000).

Reverse transcription-PCR (RT-PCR) was carried out with 50ng of total RNA, with the OneStep RT-PCR kit (Qiagen), according to the supplier's instructions, using oligonucleotides X2 and X7. As an independent control, the 16S rRNA-specific primers 16sF and 16sR were used. Prior to RT-PCR, all RNA samples were treated with Turbo DNA free Kit (Ambion). Control experiments, run in the absence of reverse transcriptase, yielded no product.

Electrophoretic mobility shift assays

All the fragments used in the electrophoretic mobility shift assays (EMSA) experiments were generated by PCR and were radioactively labeled at their 5'-end. For this purpose the reverse primer in each PCR reaction was previously end-labeled with [^{32}P]-γ-ATP using T4 polynucleotide kinase. PCR reactions were carried out using genomic DNA from *E. coli* MG1693 as template. Four different substrates were obtained with different primer pairs: bolAFw and

RNM012; bolAFw and RblrealT; P2 and RblrealT; FblrealT and RbrealT. The resulting PCR fragments were run in a 5% non-denaturing polyacrylamide (PAA) gel and purified by the crush and soak method previously described (Sambrook and Russell, 2001). The concentration of the purified fragments was measured in a Biophotometer Plus (Eppendorf).

Binding reactions were performed in a total volume of 10μl containing EMSA buffer (10mM Tris–HCl pH8, 10mM MgCl2, 100mM NaCl, 10mM KCl, 0.5mM DTT, 5% glycerol), 1nM of labeled substrate and increasing concentrations of purified H-NS. H-NS was diluted to the desired concentrations prior to the assay in 2mM Tris-HCl pH8, 0.2mM DTT, 10mM KCl and 10mM NaCl. In all the assays a control reaction without protein was performed. The binding reactions were incubated at room temperature for 20min and the samples were then analysed in a 5% non-denaturing PAA gel. DNA–protein complexes were detected using the PhosphorImager system from Molecular Dynamics.

DNaseI Footprinting

DNaseI footprinting assays were performed as described by Leblanc and Moss (Moss, 2001) with some modifications. Briefly, the DNA-protein complexes obtained as described above (but in a total volume of 50bolAp1μl), were supplemented with a cofactor solution (5mM CaCl2, 10mM MgCl2) and 5x10-3Kunitz units/μL of DNaseI, and incubated 2min at room temperature. The digestion reaction was stopped with addition of stop buffer (1% SDS, 200mM NaCl, 20mM EDTA, pH8.0) followed by phenol-chloroform-isoamylalcohol (Sigma) extraction of the digested DNA. The extracted DNA was resuspended in formamide dye mix [95% deionized formamide, 0.025% (w/v) bromophenol blue, 0.025% (w/v) xylene cyanol FF, 5mM EDTA pH8.0, 0.025% (w/v) SDS], resolved in a 8% denaturing 8.3M urea PAA sequencing gel at 1500V in 1X TBE. Digested

fragments were detected using a PhosphorImager system from Molecular Dynamics. M13 sequencing reaction was performed with Sequenase Version 2.0 sequencing kit according to the instructions manual and resolved in the same gel.

DNA curvature analysis

The online available DNA Curvature Analysis software (http://www.lfd.uci.edu/~gohlke/dnacurve/) was used with AA Wedge algorithm. This bioinformatics tool enables the compilation of the curvature values and the calculation of the global 3D structure of a DNA molecule from its nucleotide sequence. This program was used to obtain the 3D model of the bolA promoter region.

Acknowledgments

The authors would like to thank Ana Barbas for the technical support in the performed EMSA assays and Patrick Freire for the critical reading of the manuscript. This work was supported by Fundação para a Ciência e Tecnologia (FCT) (Portugal). Ricardo Moreira was supported by a Doctoral fellowship from FCT and Clémentine Dressaire and Susana Domingues were recipients of Postdoctoral fellowships, also from FCT.

References

Aldea, M., T. Garrido, C. Hernandez-Chico, M. Vicente, and S.R. Kushner. 1989. Induction of a growth-phase-dependent promoter triggers transcription of bolA, an Escherichia coli morphogene. *Embo J*. 8:3923-3931.

Aldea, M., C. Hernandez-Chico, A.G.d.l. Campa, S.R. Kushner, and M. Vicente. 1988. Identification, Cloning, and Expression of bolA, an ftsZ-Dependent Morphogene of Escherichia coli. *Journal of Bacteriology*. 170:5169-5176.

Arraiano, C.M., J.M. Andrade, S. Domingues, I.B. Guinote, M. Malecki, R.G. Matos, R.N. Moreira, V. Pobre, F.P. Reis, M. Saramago, I.J. Silva, and S.C. Viegas. 2010. The critical role of RNA processing and degradation in the control of gene expression. *FEMS Microbiol Rev*. 34:883-923.

Baba, T., T. Ara, M. Hasegawa, Y. Takai, Y. Okumura, M. Baba, K.A. Datsenko, M. Tomita, B.L. Wanner, and H. Mori. 2006. Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. *Molecular Systems Biology*. 2.

Barth, M., C. Marschall, A. Muffler, D. Fischer, and R. Hengge-Aronis. 1995. Role for the histone-like protein H-NS in growth phase-dependent and osmotic regulation of sigma S and many sigma S-dependent genes in Escherichia coli. *J Bacteriol*. 177:3455-3464.

Bertin, P., E. Terao, E.H. Lee, P. Lejeune, C. Colson, A. Danchin, and E. Collatz. 1994. The H-NS protein is involved in the biogenesis of flagella in Escherichia coli. *J Bacteriol*. 176:5537-5540.

Dorman, C.J. 2004. H-NS: a universal regulator for a dynamic genome. *Nat Rev Microbiol*. 2:391-400.

Fang, F.C., and S. Rimsky. 2008. New insights into transcriptional regulation by H-NS. *Curr Opin Microbiol*. 11:113-120.

Freire, P., J.D. Amaral, J.M. Santos, and C.M. Arraiano. 2006a. Adaptation to carbon starvation: RNase III ensures normal expression levels of bolAp1 mRNA and sigma(S). *Biochimie*. 88:341-346.

Freire, P., R. Neves Moreira, and C.M. Arraiano. 2009. BolA inhibits cell elongation and regulates MreB expression levels. *J Mol Biol*. 385:1345-1351.

Freire, P., H.L. Vieira, A.R. Furtado, M.A. de Pedro, and C.M. Arraiano. 2006b. Effect of the morphogene bolA on the permeability of the Escherichia coli outer membrane. *FEMS Microbiol Lett*. 260:106-111.

Gentry, D.R., V.J. Hernandez, L.H. Nguyen, D.B. Jensen, and M. Cashel. 1993. Synthesis of the Stationary-Phase Sigma-Factor Sigma(S) Is Positively Regulated by Ppgpp. *Journal of Bacteriology*. 175:7982-7989.

Guinote, I.B., Rute Gonçalves Matos, Patrick Freire, and Cecília Maria Arraiano. 2011. BolA Affects Cell Growth, and Binds to the Promoters of Penicillin-Binding Proteins 5 and 6 and Regulates Their Expression. *Journal of Microbiology and Biotechnology*. *in press*.

Lang, B., N. Blot, E. Bouffartigues, M. Buckle, M. Geertz, C.O. Gualerzi, R. Mavathur, G. Muskhelishvili, C.L. Pon, S. Rimsky, S. Stella, M.M. Babu, and A. Travers. 2007. High-affinity DNA binding sites for H-NS provide a molecular basis for selective silencing within proteobacterial genomes. *Nucleic Acids Res*. 35:6330-6337.

Lange, R., and R. Hengge-Aronis. 1991. Growth phase-regulated expression of bolA and morphology of stationary-phase Escherichia coli cells are controlled by the novel sigma factor sigma S. *J Bacteriol*. 173:4474-4481.

Moss, T. 2001. DNA–Protein Interactions, Principles and Protocols (Methods in Molecular Biology). *Humana Press*.

Mujacic, M., and F. Baneyx. 2006. Regulation of Escherichia coli hchA, a stress-inducible gene encoding molecular chaperone Hsp31. *Mol Microbiol*. 60:1576-1589.

Nguyen, L.H., and R.R. Burgess. 1997. Comparative analysis of the interactions of Escherichia coli sigma S and sigma 70 RNA polymerase holoenzyme with the stationary-phase-specific bolAp1 promoter. *Biochemistry*. 36:1748-1754.

Sambrook, J., and D. Russell. 2001. Molecular Cloning, A Laboratory Manual, 3rd Edition. *Cold Spring Harbor Laboratory, NY*.

Santos, J.M., P. Freire, F.S. Mesquita, F. Mika, R. Hengge, and C.M. Arraiano. 2006. Poly(A)-polymerase I links transcription with mRNA degradation via sigmaS proteolysis. *Mol Microbiol*. 60:177-188.

Santos, J.M., P. Freire, M. Vicente, and C.M. Arraiano. 1999. The stationary-phase morphogene bolA from Escherichia coli is induced by stress during early stages of growth. *Mol Microbiol*. 32:789-798.

Santos, J.M., M. Lobo, A.P. Matos, M.A. De Pedro, and C.M. Arraiano. 2002. The gene bolA regulates dacA (PBP5), dacC (PBP6) and ampC (AmpC), promoting normal morphology in Escherichia coli. *Mol Microbiol*. 45:1729-1740.

Sette, M., R. Spurio, E. Trotta, C. Brandizi, A. Brandi, C.L. Pon, G. Barbato, R. Boelens, and C.O. Gualerzi. 2009. Sequence-specific Recognition of DNA by the C-terminal Domain of Nucleoid-associated Protein H-NS. *Journal of Biological Chemistry*. 284:30453-30462.

Vieira, H.L., P. Freire, and C.M. Arraiano. 2004. Effect of Escherichia coli morphogene bolA on biofilms. *Appl Environ Microbiol*. 70:5682-5684.

White-Ziegler, C.A., and T.R. Davis. 2009. Genome-wide identification of H-NS-controlled, temperature-regulated genes in Escherichia coli K-12. *J Bacteriol*. 191:1106-1110.

Wood, T.K., A.F. Gonzalez Barrios, M. Herzberg, and J. Lee. 2006. Motility influences biofilm architecture in Escherichia coli. *Appl Microbiol Biotechnol*. 72:361-367.

Yamada, H., S. Muramatsu, and T. Mizuno. 1990. An Escherichia coli protein that preferentially binds to sharply curved DNA. *J Biochem*. 108:420-425.

Yamamoto, K., R. Nagura, H. Tanabe, N. Fujita, A. Ishihama, and R. Utsumi. 2000. Negative regulation of the bolAp1 of Escherichia coli K-12 by the transcription factor OmpR for osmolarity response genes. *Fems Microbiology Letters*. 186:257-262.

Supplementary Data

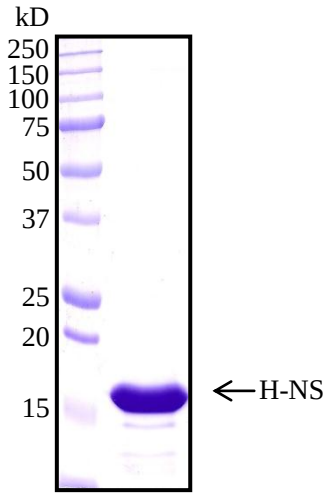

Fig. S1. SDS-PAGE analysis of purified H-NS.
Protein sample was visualized by Coomassie blue staining. Molecular weight marker (Precision Plus Protein Prestained Standards-Bio-Rad) is shown on the left side of the image. Purified protein was separated on a 13.5 % polyacrylamide gel (17.7 kDa).

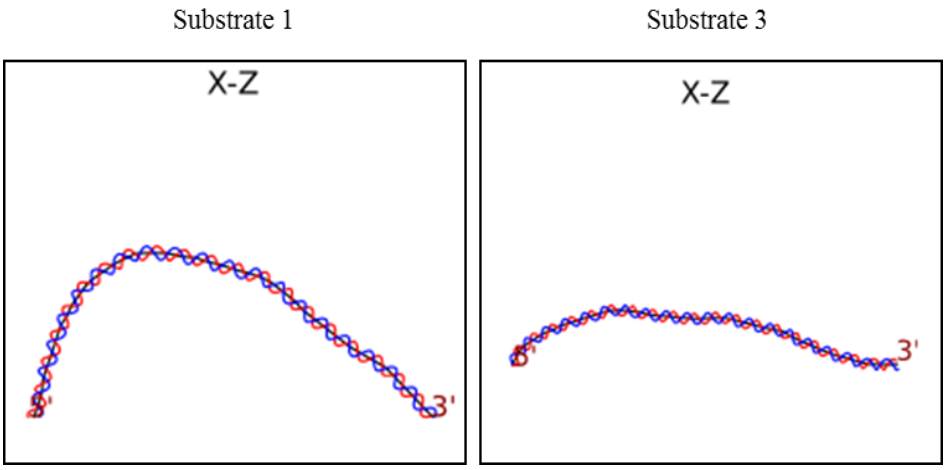

Fig. S2. DNA Curvature Analysis.
DNA conformation for substrate 1 and substrate 3 (on top of the images) was analyzed using DNA Curvature Analysis bioinformatics tool according to the AA Wedge model.

Chapter 3

BolA Inhibits Cell Elongation and Regulates MreB Expression Levels

This chapter contains data published in:
Freire, P., **R.N. Moreira**, and C.M. Arraiano. 2009. BolA inhibits cell elongation and regulates MreB expression levels**. J Mol Biol. 385:1345-1351**

Abstract

The morphogene *bolA* is a general stress response gene in *Escherichia coli* that induces a round morphology when overexpressed. Results presented in this report show that increased BolA levels can inhibit cell elongation mechanisms. MreB polymerization is crucial for the bacterial cell cytoskeleton, and this protein is essential for the maintenance of a cellular rod shape. In this report, we demonstrate that *bolA* overexpression affects the architecture of MreB filaments. An increase in BolA leads to a significant reduction in MreB protein levels and *mreB* transcripts. BolA affects the *mreBCD* operon *in vivo* at the level of transcription. Furthermore, our results show that BolA is a new transcriptional repressor of MreB. The alterations in cell morphology induced by *bolA* seem to be mediated by a complex pathway that integrates PBP5, PBP6, MreB, and probably other regulators of cell morphology/elongation.

Introduction

Penicillin-binding proteins (PBPs) are key players in cell elongation and division mechanisms (Spratt and Pardee, 1975). In *Escherichia coli*, PBP2 is responsible for lateral murein extension, leading to cell elongation, while PBP3 is specific for septal murein production during cell division. Specific inhibition of PBP2 by mecillinam causes *E. coli* to grow as spherical cells, while inhibition of PBP3 using aztreonam blocks septal peptidoglycan synthesis, leading to a characteristic filamented cell phenotype (Spratt and Pardee, 1975). PBP2 is essential for cell elongation. MreB is a structural homolog of actin that is also essential for cell elongation and maintenance of a bacterial rod shape (Jones et al., 2001; van den Ent et al., 2001). The morphogene *bolA* induces a spherical shape when overexpressed, and it has been established as a general stress response gene (Santos et al., 1999). The expression of *bolA* is tightly controlled (Freire et al., 2006a; Lange and Hengge-Aronis, 1991; Santos et al., 2006). *bolA* increases biofilm formation (Vieira et al., 2004) and modulates cell permeability (Freire et al., 2006b); in addition, it was demonstrated that *bolA* increases the expression of PBP5, PBP6 and ampC mRNA (Aldea et al., 1988; Santos et al., 2002). In this work, we analysed the effect of BolA on cell growth and elongation using a set of specific antibiotics that induce known morphology alterations through the inhibition of PBPs. Results show that BolA inhibits the mechanism of cell elongation and can act as a new transcriptional repressor of MreB expression.

Results & Discussion

BolA expression affects growth rate and cell elongation

Plasmid pPFA02 was constructed by cloning the *bolA* coding region in-frame with a (His)6 tag at the 5′ end in a pET28a plasmid under the control of a LacZ promoter (Novagen). CMA50 is a BL21(DE3) strain (Novagen) transformed with pPFA02 plasmid. High expression of (His)6–BolA was achieved 30 min after 1 mM IPTG (Merck) was added to the growth medium. Microscopic phase-contrast observations showed that all cells became round or olive-shaped after 1 h of induction (Supplementary Fig. S1), demonstrating that overexpression of (His)6–BolA induces the same cellular morphology alterations that are observed when the native BolA protein is overexpressed (Santos et al., 1999). Two hours after induction of BolA, the optical density at 620nm (OD_{620}) of the culture increased 2.5X; without induction of *bolA*, the OD_{620} increased 7X (data not shown). The overexpression of BolA appears to be sufficient to retard cell growth rate. Aztreonam is a specific inhibitor of PBP3 activity that prevents septation and induces the formation of cellular filaments (Spratt and Pardee, 1975). Exponentially growing cells were regular rod-shaped bacteria, but some filamenting cells were also visible (about 2% of the total cell population) (Fig. 1a). Addition of aztreonam induced cell filamentation, as expected (Fig. 1a1 and a2). When *bolA* expression was induced after aztreonam addition, cells remained shaped as filaments (Fig. 1b1 and b2). However, these filaments no longer increased in length. Elongation seemed to be arrested. Surprisingly, after 90 min of *bolA* overexpression, a branched phenotype arose (Fig. 1b2). However, when *bolA* was induced in exponential phase and aztreonam was added 30 min later, cells no longer became filaments as could be expected due to aztreonam effects and furthermore acquired a shorter morphology (Fig. 1c1 and c2). Even though

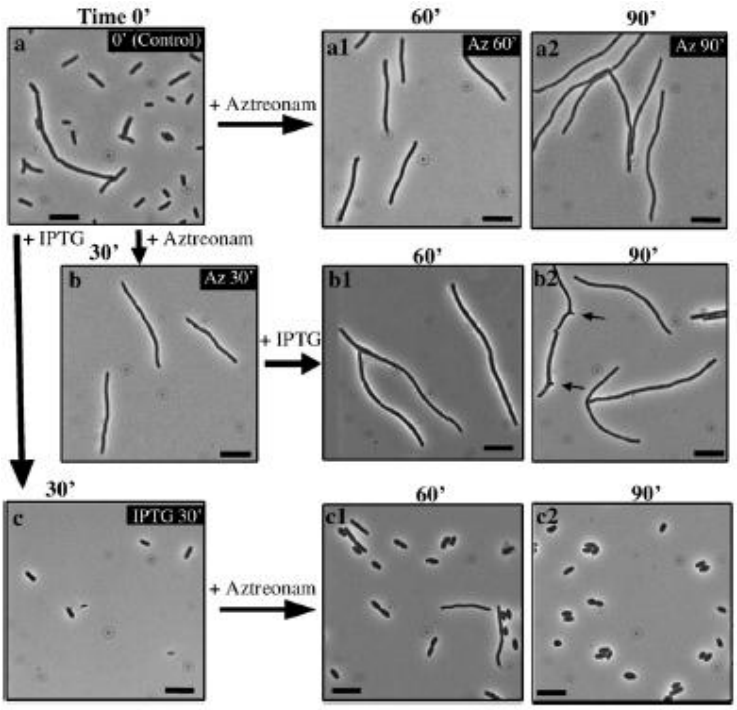

Fig. 1. Phase-contrast microscopy photographs. Batch cultures grown aerobically in LB medium at 37 °C and 120 rpm were launched from overnight cultures, diluted to an OD_{620} of 0.08 (Santos et al., 1999). Cells were harvested and fixed onto slides coated with a 1% agarose film (Donachie et al., 1976). Images were obtained using a DMRB microscope (Leica) under phase-contrast optics coupled to a CCD camera, with Leica software. CMA50 strain morphology alterations were observed in LB medium after addition of aztreonam (20 μg/mL) or IPTG (1 mM) starting at an OD_{620} of 0.4. (a) Exponentially growing CMA50 in LB medium. Time 0′ for aztreonam (Az) addition or IPTG addition. (a1) Sixty minutes after aztreonam addition to the medium. (a2) Ninety minutes after aztreonam addition. (b) Addition of IPTG to induce *bolA* expression 30 min after aztreonam treatment. (b1) Sixty minutes after aztreonam treatment and 30 min after *bolA* induction. (b2) Ninety minutes after aztreonam addition and 60 min after *bolA* induction. Black arrows show the beginning of cell branching. (c) Thirty minutes after induction of *bolA* by IPTG; time 0′ for addition of aztreonam. (c1) Sixty minutes after *bolA* induction and 30 min after aztreonam treatment. (c2) Ninety minutes after *bolA* induction and 60 min of aztreonam addition. Glucose (0.4% w/v) was added to the medium in control experiments to ensure complete shutdown of the expression of pPFA02 (data not shown). The levels of (His)6–BolA were determined by quantitative Western blot and are supplied as supplementary data (Fig. S2). Black bar represents 5 μm.

some longer cells were still detected 30 min after aztreonam addition (Fig. 1c1), the population eventually reached 100% of short cells (Fig. 1c2). The levels of (His)6–BolA in all the conditions observed by microscopy were determined by quantitative Western blot (Supplementary Fig. S2). Detection was performed with an anti-(His) antibody from GE Healthcare at a concentration of 1:5000. The intensity of the bands measured normalized by measurements of elongation factor (EF)-Tu protein as internal control. The levels of BolA were maintained with slight variations throughout all conditions. This ensured that BolA is produced in considerable levels whenever it is induced. Conversely, when no induction with IPTG was performed, no level of the protein was detected. Thus, BolA overexpression can prevent elongation of the cell. The absence of *bolA*-mediated morphology when septation is inhibited before *bolA* induction (Fig. 1b1 and b2) indicates two possibilities: (a) the cells might be committed to an irreversible morphological pathway by the influence of aztreonam and BolA is no longer able to induce a rounder shape or (b) BolA-dependent inhibition of elongation might require a functional septation machinery, here inhibited by blocking PBP3, at least in an initial phase. Furthermore, the longer cells observed in Fig. 1c1 cannot become shorter in Fig. 1c2 by dividing since septation is inhibited. Therefore, the longer cells either might have been dying by lysis or were somehow being shortened by the overexpression of *bolA*.

BolA prevents cellular elongation/rod shape maintenance mechanisms

Cefmetazole is a cephalosporin that inhibits all E. coli PBPs except PBP2 (Ohya et al., 1978). This antibiotic was used simultaneously with aztreonam in a similar experiment as in Fig. 1 to inhibit all PBP functions, except for PBP2, and focus the analysis of the effect of *bolA* on elongation mechanisms, independently of PBP5 or PBP6, previously shown to be regulated by BolA (Santos et al., 2002).

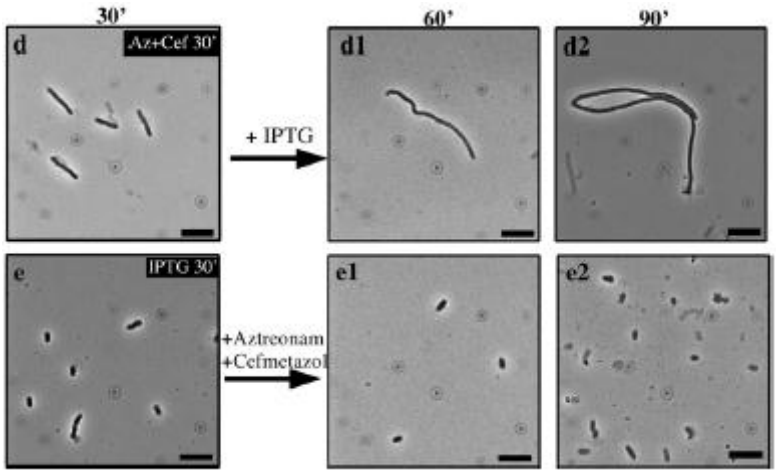

Fig. 2. Phase-contrast microscopy photographs. CMA50 strain morphology alterations were observed in LB medium after addition of aztreonam (20 μg/mL) plus cefmetazole (1 μg/mL) (cef) or IPTG starting at an OD620 of 0.4. Time 0′ of the experiment corresponds to the photo in Fig.1a. (d) Thirty minutes after aztreonam+cefmetazole addition to the medium; time 0′ for addition of IPTG to induce *bolA* expression. (d1) Sixty minutes after aztreonam+cefmetazole treatment and 30 min after *bolA* induction. (d2) Ninety minutes after aztreonam+cefmetazole addition and 60 min after *bolA* induction. (e) Thirty minutes after induction of *bolA* by IPTG; time 0′ for addition of aztreonam+cefmetazole. (e1) Sixty minutes after *bolA* induction and 30 min after aztreonam+cefmetazole treatment. (e2) Ninety minutes after *bolA* induction and 60 min after aztreonam+cefmetazole addition. The levels of (His)6–BolA were determined by quantitative Western blot and are supplied as supplementary data (Fig. S2). Black bar represents 5 μm.

The results were generally the same as those illustrated in Fig. 1, showing that *bolA* overexpression is unable to revert the filament morphology when septation is blocked before its own induction (Fig. 2d1 and d2) and that cells are unable to elongate when *bolA* is overexpressed prior to septation inhibition (Fig. 2e1 and e2). However, the elongation now observed in Fig. 2d1 and d2 is strictly related to PBP2 activity, among all PBPs. It is interesting to verify that *bolA* can affect morphology independently of PBP5 and/or PBP6. Therefore, BolA overexpression either blocks PBP2-dependent cell elongation or affects another mechanism involved in the normal maintenance of the rod shape and essential for elongation.

Overexpression of PBP2 was shown to be unable to revert the round phenotype caused by *bolA* overexpression back to a bacilli shape (Aldea et al., 1988). BolA might then be affecting other elements involved in cell elongation mechanisms that indirectly impair PBP2-dependent cell elongation. A good candidate is MreB, a structural homolog of actin essential for cell elongation and maintenance of the rod shape (Jones et al., 2001; van den Ent et al., 2001). A possible correlation can also be established between *bolA*-induced round morphology and the spherical cells caused by mutations of the *mreB* gene or specific inhibition of MreB (van den Ent et al., 2001).

BolA affects the architecture of MreB filaments

Immunofluorescence experiments to detect MreB filaments were performed in order to check for any influence of *bolA* on their spatial arrangement (Fig. 3.1). MreB polymerizes to form a spiralled structure along the interior of the cell wall (Jones et al., 2001). MreB polymers forming the cytoskeleton were clearly visible when BolA was not overexpressed (Fig. 3.1a–d). When the expression levels of BolA increased, no more MreB filaments can be detected and the signal was spread all over the spherical cell (Fig. 3.1h–j). MreB filaments nevertheless remained detectable by immunofluorescence in round cells caused by addition of mecillinam, an inhibitor of PBP2 (Fig. 3.1e–g), showing that loss of MreB localization under *bolA* overexpression is not due to the shape alteration from rod to sphere. A similar observation was made when MreB polymerization was inhibited by A22, a specific inhibitor of MreB (Karczmarek et al., 2007). Therefore, the results obtained show that BolA overexpression affects MreB filaments spatial organization. MreB was induced with IPTG from plasmid pTK51214 in several conditions and strains (data not shown) in an attempt to rescue the *bolA* spherical morphology. No reversion of cell morphology could be detected. BL21+pPFA02 strain was co-transformed with pTK512 to further study these effects. MreB and

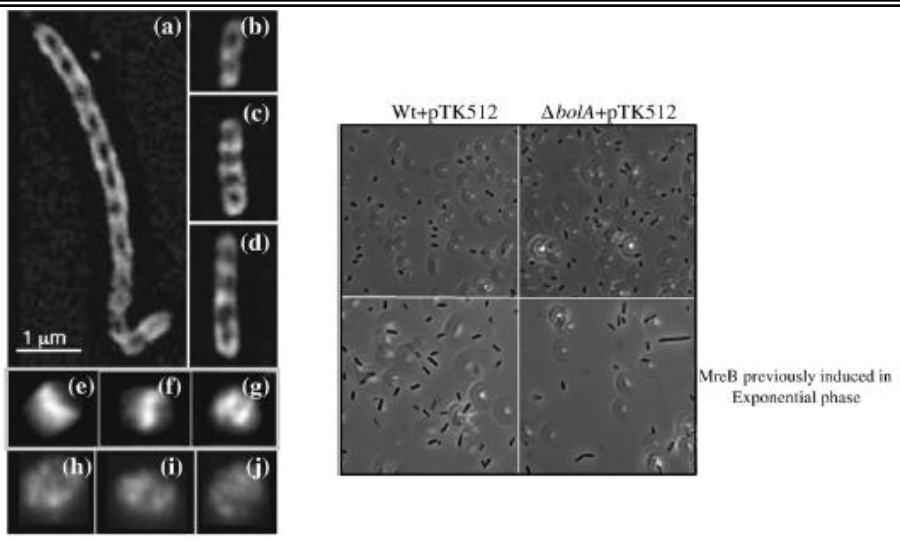

Fig. 3. (1) Analysis of *E. coli* cytoskeleton by immunofluorescence microscopy. Anti-MreB antibodies (Kruse et al., 2003) were used at a 1:100 dilution; secondary TRITC anti-rabbit (Sigma), at 1:300. Cells were fixed in phosphate buffered saline 1X, 4% formaldehyde, and 0.02% glutaraldehyde (Harry et al., 1995). Cells were permeabilized with lysozyme (10 $mg.mL^{-1}$) and applied to polylysine pretreated coverslips, fixed with methanol and acetone, and blocked with 2% bovine serum albumin and 0.05% Tween-20 in phosphate-buffered saline 1X. Images collected by immunofluorescence in a DMRB microscope (Leica) were treated with a deconvolution filter from MetaMORPH software. (a–d) Exponentially growing CMA50 cells showing the helical structures corresponding to the polymers of MreB forming the cytoskeleton. Subpanel (a) corresponds to an example of the filaments observed in these conditions. (e–g) Control experiment showing the visualization of MreB-defined filaments in round cells treated with mecillinam. (h–j) Overexpression of BolA 2 h after IPTG addition. Control experiments rule out any influence of glucose or IPTG on the morphology alterations (data not shown). The levels of (His)6–BolA were determined by quantitative Western blot and are supplied as supplementary data (Fig. S2). (2) Phase-contrast microscopy photographs. Batch cultures grown aerobically in LB medium at 37 °C and 120 rpm were launched from overnight cultures, diluted to an OD_{620} of 0.08 (Santos et al., 1999). Cells were harvested and fixed onto slides coated with a 1% agarose film (Donachie et al., 1976). Images were obtained using a DMRB microscope (Leica) under phase-contrast optics coupled to a CCD camera, with Leica software. MG1693 and an isogenic *ΔbolA* were transformed with pTK51214 plasmid that overexpresses the *mreBCD* operon with IPTG. The upper panel shows their morphology in the stationary phase of growth without induction. The lower panel shows the morphology alterations observed in the stationary phase when *mreBCD* is induced previously in the exponential phase of growth.

(His)6–BolA were induced simultaneously with 1 mM IPTG. Overexpression of BolA and MreB together gave a mixed cell phenotype (round, lemon-shaped cells and rods), but, in general, longer cells were obtained due to the opposite effect of MreB in cell morphology (Supplementary Fig. S1). After 2 h of induction, when only BolA was overexpressed in the BL21+pPFA02 strain, cells became spherical or lemon-shaped. Non-induced cells presented the typical phenotype of BL21: rods with some filaments. MreB overexpression seems to reduce the impact of BolA in cell morphology. MG1693 and an isogenic *bolA* deletant were also transformed with pTK512 and studied in stationary phase to assess this effect on other strains. As above, after a rounder morphology was established, the induction of MreB was unable to restore longer cells. However, when MreB was induced in the exponential phase, it prevented the formation of shorter cells observed in the stationary phase. This effect is even more visible in *bolA* deletant strain, showing that MreB has an effect opposite to the influence of BolA in cell morphology (Fig. 3.2). Even though MreB overexpression cannot reverse the morphology induced by BolA, higher levels of MreB in the cell clearly impair the induction of a rounder/shorter shape by BolA.

BolA affects MreB expression levels

The process of MreB filament formation is probably dependent on the intracellular levels of MreB, in a way similar to what happens in the actin polymerization process (Korn et al., 1987). Therefore, if *bolA* significantly lowers the expression levels of MreB, this could be interfering with the architecture of MreB polymers. Western blots were performed to assess variations of MreB protein levels related to *bolA* overexpression. The results show that MreB protein levels were reduced by threefold when BolA was overexpressed (Fig. 4a). The detection of EF-Tu by specific antibodies on the same membranes shows that the variations observed are not due to pleiotropic effects of IPTG or BolA overexpres-

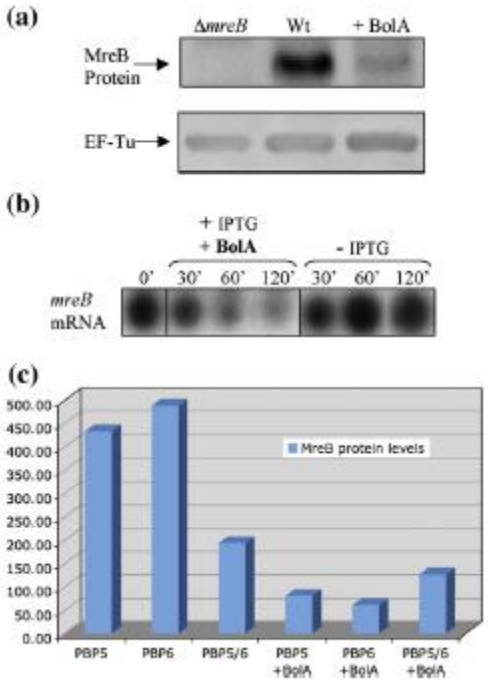

Fig. 4. (a) Western blot showing the levels of MreB in the cell. Bacterial proteins were extracted using Bugbuster (Novagen). Quantification was according to the Lowry method, and equal amounts of total protein were loaded in 12% SDS-PAGE gels (Sambrook and Russell, 2001). After transfer, membranes were incubated with anti-MreB antibodies at a dilution of 1:10,000 (Kruse et al., 2003). MC1000*ΔmreB* strain was used as negative control (Kruse et al., 2003). The top lane is *ΔmreB*, the middle lane shows the levels of this protein with basal expression of BolA, and the bottom lane shows the levels of MreB upon overexpression of BolA. EF-Tu detection was used as a control of total protein quantification. (b) Representative dot blot showing the analysis of steady state *mreB* mRNA levels in CMA50 strain after overexpression of *bolA*. Total RNA was extracted as previously described (Santos et al., 2006). Equal amounts of total RNA were blotted onto Hybond+ membranes (GE) and fixed by UV light. An *mreB* DNA probe spanning the entire *mreB* ORF was obtained by PCR using Taq polymerase (Roche) and the primers MreB1 (5′-attgacctgggtactgcg-3′) and MreB2 (5′-ctcttcgctgaacaggtc-5′) produced by STABVida. Membranes were hybridized and washed as described previously (Miller, 1992). Membranes were autoradiographed using Biomax MR from Kodak, and bands were quantified with an IMAGEQUANT™ densitometer (Molecular Dynamics). 0′ represents *mreB* mRNA levels at an OD_{620} of 0.4. The levels of (His)6–BolA were determined by quantitative Western blot and are supplied as supplementary data (Fig. S3). (c) MreB protein levels in PBP5, PBP6, and double PBP5/PBP6 mutants (Santos et al., 2002) in LB medium stationary phase. Quantifications were done by Western blot and normalized by EF-Tu determination on the same membranes. Lanes 1–3 show the results for the strains without *bolA* overexpression, while lanes 4–6 show MreB levels in the same conditions but with overexpression of *bolA* using plasmid pMAK580 (containing *bolA* with native promoters) as described previously (Santos et al., 2002).

sion. BolA is thus shown to act as a new negative regulator of MreB. By interfering with the levels of MreB, BolA impairs the stability of the cytoskeleton in *E. coli*. The disruption of the internal cell scaffold could furthermore explain how cells could eventually shorten from a longer rod shape (Fig. 1c1) to olive-shaped cells (Fig. 1c2) when septation is inhibited. RNA dot-blot experiments were performed to check whether the change in MreB protein levels derives from a reduction in *mreB* mRNA levels (Fig. 4b).The results show that induction

of BolA levels was indeed able to significantly reduce the levels of *mreB* transcripts in less than 60 min. Therefore, *bolA* represses the levels of *mreB* mRNA. The levels of induced BolA were determined by quantitative Western blot and are provided as supplementary data (Fig. S3). The results obtained were further confirmed by quantitative reverse-transcription PCR. A Transcriptor First Strand cDNA Synthesis Kit (Roche) was used to reverse transcribe total RNA to cDNA using the random hexamer primer following the manual protocol. Quantitative PCRs were performed to amplify *mreB* and 16S RNA cDNAs using the primers RT-MREB (5′-acttgtccattgacctgggtactg-3′) and RT-MREB2 (5′-gccgccgtgcatgtcgatcatttc-3′) and the primers 16S rRNA F (5′-aga gtt tga tcc tgg ctc ag- 3′) and 16S rRNA R (5′-acg gct acc ttg tta cga ctt-3′), respectively. Equal amounts of the sample were loaded in 0.7% agarose gel (Supplementary Fig. S4). Quantification was done with ImageJ software and normalized by 16S RNA cDNA levels. The results obtained from three replicated experiments indicate intensities of 0.82±0.12 for the *mreB* cDNA band 60 min after induction of BolA and 1.45±0.22 without any induction. BolA therefore reduces the levels of *mreB* RNA to about 55% of their normal levels.

BolA represses operon mreBCD transcription by direct binding to its promoters

A plasmid pRMA1 was constructed containing the *gfp* gene encoding green fluorescent protein under the control of the promoters of the *mreBCD* operon using vector p363 (Miksch and Dobrowolski, 1995). Total protein was extracted as above, with and without overexpression of BolA, and green fluorescent protein fluorescence was quantified in a Varian-Eclipse fluorescence spectrophotometer. The data obtained were normalized per cell by quantifying EF-Tu protein present in the different protein extracts. The fluorescence per cell (+BolA)/fluorescence per cell (wt) ratio, representing the variations in *mreBCD*

transcription by overexpression of *bolA*, were determined in BL21+pPFA02 strain after 1h of induction. The average ratio obtained was 0.64±0.04. BolA overexpression is therefore able to shut down transcription of *mreBCD* operon *in vivo* to about 64% of its normal expression, in correlation with the levels of *mreB* RNA detected above. (His)6–BolA was purified by histidine affinity chromatography using HiTrap chelating HP columns and an AKTA HPLC system (GE Healthcare). Purity of the protein was verified by SDS-PAGE. The purified protein was immobilized by amine coupling in a CM5 sensor chip on a Biacore 2000 system (GE Healthcare) following the manufacturer's instructions and analysed by surface plasmon resonance. Biosensor assays were run at 25 °C in buffer containing 20 mM Tris–HCl, pH 8, 100 mM KCl, 1 mM dithiothreitol, and 25 mM ethylenediaminetetraacetic acid. Operon *mreBCD* promoters and *bolA* open-reading frame (ORF) DNA encoding fragments were amplified by PCR using the primers mreB1 (5′-gccacttgatactaacgtg-3′) and mreB2 (5′-ggggcggaaaagaaaatc-3′) and the primers bolAX2 (5′-gtcacaatgtcccagccg-3′) and bolAX9 (5′-ccagacaaaacaaaacggcccg-3′), respectively. The amplified DNA fragments were injected as ligands. All experiments included replicate injections of six concentrations of each DNA sequence (ranging from 0 to 3 pM). Dissociation constants (K_D's) were calculated using the BIA Evaluation 3.0 software package, according to the fitting model 1:1 Langmuir binding. We determined a K_D of 6.9±2.4 nM for BolA interaction with *mreB* promoters and a K_D of 23.6±5.4 nM for the interaction with the *bolA* ORF. The K_D of (His)6–BolA interaction with the *bolA* ORF sequence is therefore 3.5-fold higher than that with the *mreBCD* promoter sequence, showing that BolA has a significantly higher affinity for the promoter sequence of *mreBCD*. BolA is thus able to bind directly with high affinity to the promoter sequence of *mreB* and therefore acts as a new transcriptional repressor of MreB expression levels. MreB concentration in fast-growing cells reaches 40,000 molecules/cell; in slow-growing cells, it was

estimated at 17,000 molecules/cell (Kruse et al., 2003). Inversely, *bolA* mRNA levels are low in fast-growing cells but increase by about 20-fold in slow-growing cells (Santos et al., 1999); the regulation of BolA expression might therefore be connected to the differential expression of MreB during different growth phases.

BolA plays a central role in a morphogenetic pathway including PBP5, PBP6, and MreB

BolA induces the expression levels of PBP5 and PBP6 at the onset of the exponential phase (Santos et al., 2002). BolA overexpression was also shown to be unable to promote a round morphology in a PBP5/PBP6 double mutant (Santos et al., 2002). The inhibition of all PBPs except PBP2 in Fig. 2e1 and e2 shows that *bolA* is nevertheless able to induce a shorter olive-shaped morphology independently of PBP5 or PBP6. A similar reduction in cell length was also reported in the PBP5/PBP6 double mutant (Santos et al., 2002). BolA's effect on cell morphology alterations thus seems to be based on the integration of a complex set of regulations. The levels of MreB protein were analyzed in the PBP5 and PBP6 single and double mutants that were previously studied (Santos et al., 2002). Protein levels were normalized by quantification of EF-Tu levels (Fig. 4c). A general negative effect of *bolA* overexpression on the levels of MreB is clearly confirmed (columns 4 to 6); even though this effect is lower in the double PBP5/PBP6 mutant. MreB levels are lower in the single mutants as compared with the double mutant upon overexpression of *bolA*. This low concentration might not be enough to permit polymerization of MreB filaments, as seen in the data presented in Fig. 3. The conjugated effects of BolA in MreB, PBP5, and PBP6 thus contribute to induce the round morphology in PBP single mutants. Likewise, the absence of a round morphology induced by *bolA* in the PBP5/PBP6 double mutant could be correlated not only to the lack of PBP5 and PBP6 but also to the higher levels of MreB observed in that strain. Furthermore, since this strain shows lower

levels of MreB than the single mutants, independently of *bolA* overexpression (column 3), expression of MreB might also be influenced by PBP5 and/or PBP6. BolA therefore seems to play a central role in a complex web of regulators of cell morphology/elongation that includes PBP5, PBP6, MreB, and probably other factors. The induction of PBP5 and PBP6 and the reduction in MreB levels by BolA overexpression converge to inhibit cell elongation and induce a rounder morphology.

This work shows that *bolA*-induced cell morphology alterations are mediated by a complex pathway that integrates PBP5, PBP6, and MreB. The finding that BolA can directly repress the transcription of *mreBCD* and lower the levels of MreB in the cell presents a broad impact on cellular features, such as morphology maintenance and elongation mechanisms, especially in stress conditions when *bolA* is induced. It is also a major step toward understanding the regulation of MreB expression, a protein responsible for the cytoskeleton, an essential architectural element of the bacterial cell. Further studies will be necessary to provide more insights on these novel regulation pathways and how the different elements involved influence one another.

Acknowledgments

This work was funded by Fundação para a Ciência e a Tecnologia (Portugal). P.F. and R.N.M. are recipients of fellowships from Fundação para a Ciência e a Tecnologia. We thank Kenn Gerdes and Miguel Vicente for their kind gifts of anti-MreB serum as well as plasmid pTK512 and anti-EF-Tu serum, respectively. We also thank Gerhard Miksch for the kind gift of plasmid p363.

References

Aldea, M., C. Hernandez-Chico, A.G.d.l. Campa, S.R. Kushner, and M. Vicente. 1988. Identification, Cloning, and Expression of bolA, an ftsZ-Dependent Morphogene of Escherichia coli. *Journal of Bacteriology*. 170:5169-5176.

Donachie, W.D., K.J. Begg, and M. Vicente. 1976. Cell length, cell growth and cell division. *Nature*. 264:328-333.

Freire, P., J.D. Amaral, J.M. Santos, and C.M. Arraiano. 2006a. Adaptation to carbon starvation: RNase III ensures normal expression levels of bolAp1 mRNA and sigma(S). *Biochimie*. 88:341-346.

Freire, P., R. Neves Moreira, and C.M. Arraiano. 2009. BolA inhibits cell elongation and regulates MreB expression levels. *J Mol Biol*. 385:1345-1351.

Freire, P., H.L. Vieira, A.R. Furtado, M.A. de Pedro, and C.M. Arraiano. 2006b. Effect of the morphogene bolA on the permeability of the Escherichia coli outer membrane. *FEMS Microbiol Lett*. 260:106-111.

Harry, E.J., K. Pogliano, and R. Losick. 1995. Use of immunofluorescence to visualize cell-specific gene expression during sporulation in Bacillus subtilis. *J Bacteriol*. 177:3386-3393.

Jones, L.J., R. Carballido-Lopez, and J. Errington. 2001. Control of cell shape in bacteria: helical, actin-like filaments in Bacillus subtilis. *Cell*. 104:913-922.

Karczmarek, A., R. Martinez-Arteaga, S. Alexeeva, F.G. Hansen, M. Vicente, N. Nanninga, and T. den Blaauwen. 2007. DNA and origin region segregation are not affected by the transition from rod to sphere after inhibition of Escherichia coli MreB by A22. *Mol Microbiol*. 65:51-63.

Korn, E.D., M.F. Carlier, and D. Pantaloni. 1987. Actin polymerization and ATP hydrolysis. *Science*. 238:638-644.

Kruse, T., J. Moller-Jensen, A. Lobner-Olesen, and K. Gerdes. 2003. Dysfunctional MreB inhibits chromosome segregation in Escherichia coli. *EMBO J*. 22:5283-5292.

Lange, R., and R. Hengge-Aronis. 1991. Growth phase-regulated expression of bolA and morphology of stationary-phase Escherichia coli cells are controlled by the novel sigma factor sigma S. *J Bacteriol*. 173:4474-4481.

Miksch, G., and P. Dobrowolski. 1995. Growth phase-dependent induction of stationary-phase promoters of Escherichia coli in different gram-negative bacteria. *J Bacteriol*. 177:5374-5378.

Miller, J.H. 1992. Experiments in Molecular Genetics Cold Spring Harbor Laboratory. *Cold Spring Harbor, NY*.

Ohya, S., M. Yamazaki, S. Sugawara, S. Tamaki, and M. Matsuhashi. 1978. New cephamycin antibiotic, CS-1170: binding affinity to penicillin-binding

proteins and inhibition of peptidoglycan cross-linking reactions in Escherichia coli. *Antimicrob Agents Chemother*. 14:780-785.

Sambrook, J., and D. Russell. 2001. Molecular Cloning, A Laboratory Manual, 3rd Edition. *Cold Spring Harbor Laboratory, NY*.

Santos, J.M., P. Freire, F.S. Mesquita, F. Mika, R. Hengge, and C.M. Arraiano. 2006. Poly(A)-polymerase I links transcription with mRNA degradation via sigmaS proteolysis. *Mol Microbiol*. 60:177-188.

Santos, J.M., P. Freire, M. Vicente, and C.M. Arraiano. 1999. The stationary-phase morphogene bolA from Escherichia coli is induced by stress during early stages of growth. *Mol Microbiol*. 32:789-798.

Santos, J.M., M. Lobo, A.P. Matos, M.A. De Pedro, and C.M. Arraiano. 2002. The gene bolA regulates dacA (PBP5), dacC (PBP6) and ampC (AmpC), promoting normal morphology in Escherichia coli. *Mol Microbiol*. 45:1729-1740.

Spratt, B.G., and A.B. Pardee. 1975. Penicillin-binding proteins and cell shape in E. coli. *Nature*. 254:516-517.

van den Ent, F., L.A. Amos, and J. Lowe. 2001. Prokaryotic origin of the actin cytoskeleton. *Nature*. 413:39-44.

Vieira, H.L., P. Freire, and C.M. Arraiano. 2004. Effect of Escherichia coli morphogene bolA on biofilms. *Appl Environ Microbiol*. 70:5682-5684.

Supplementary Data

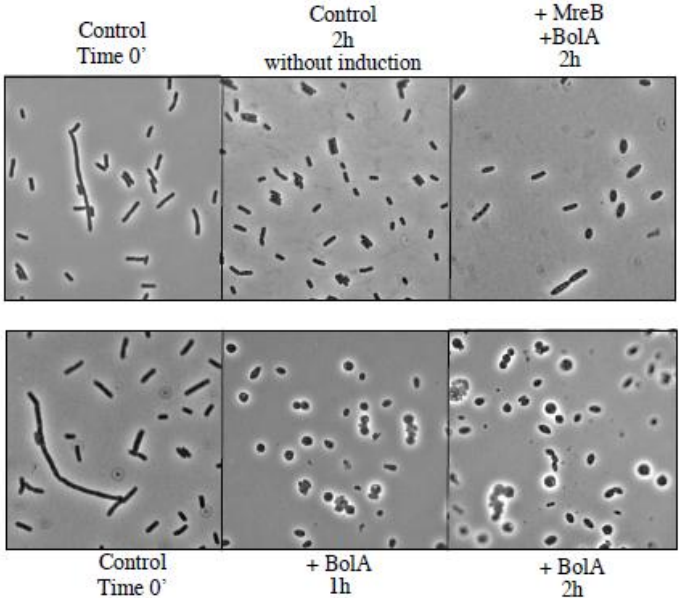

Fig. S1. Phase contrast microscoscopy photographs. Batch cultures grown aerobically in LB medium at 37°C and 120 rpm were launched from overnight cultures, diluted to an optical density of 0,08 at 620 nm (OD_{620}) (Santos et al., 1999). Cells were harvested and fixed on to slides coated with 1% agarose film (Korn et al., 1987). Images were obtained using DMRB microscope (Leica) under phase-contrast optics coupled to a CCD camera, with Leica software. The upper panel presents strain BL21+pPFA02+pTK512 with a simultaneous induction of MreB and BolA with 1mM IPTG for 2h and the control experiment without induction. Lower panel shows the morphology induced by BolA overexpression after 1 and hours with IPTG 1mM in strain BL21+pPFA02.

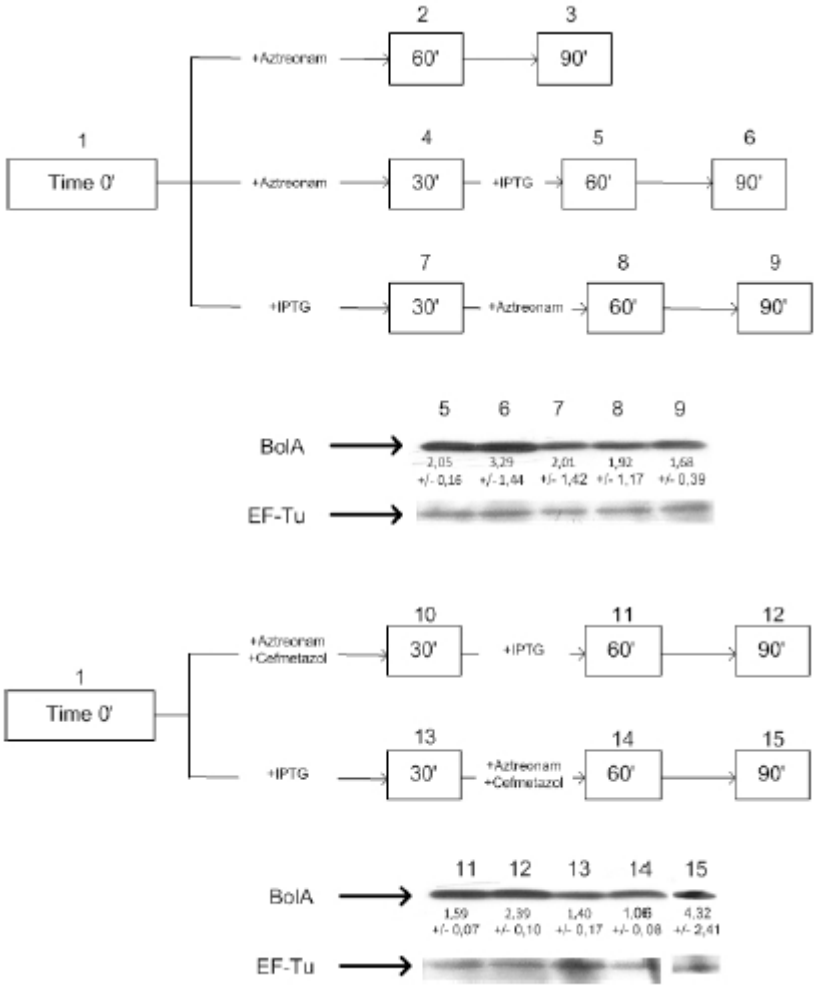

Fig. S2. Quantitative Western Blots with the levels of induced BolA in the conditions observed in Figure 1 (upper panel) and Figure 2 (lower panel). Experiments were performed as in Figure 4.A with normalization by EF-Tu levels. The numbers represent the amount of BolA detected divided by the amount of EF-Tu. The points showed in the experiment diagram without figures had no detectable levels of (His)6-BolA and correspond to non-induced conditions. Detection of (His)6-BolA was performed with an antibody anti-His from GE Healthcare at 1:5000.

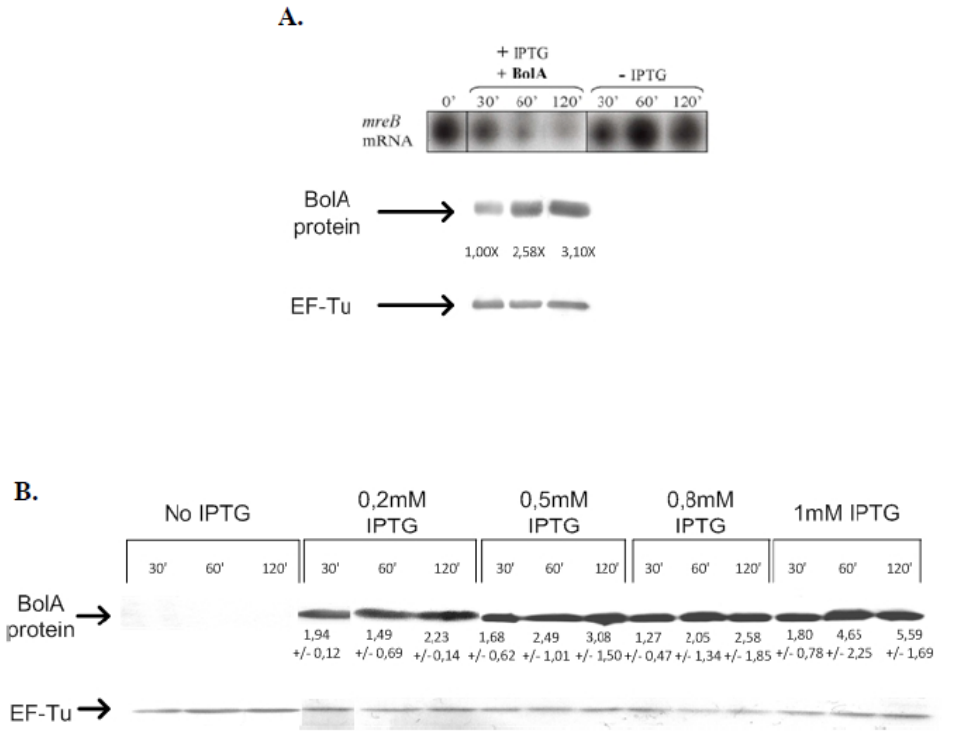

Fig. S3. Quantitative Western Blots with the levels of induced BolA in the conditions observed in Figure 3.1 and Figure 4.B (upper panel A). The intensities measured are indicated using the levels of BolA after 30′ of induction as reference. BolA is not detected without induction by IPTG. B. Analysis of induction levels of BolA under different IPTG concentrations. The numbers represent the amount of BolA detected divided by the amount of EF-Tu. Detection of (His)6-BolA was performed with an antibody anti-His from GE Healthcare at 1:5000.

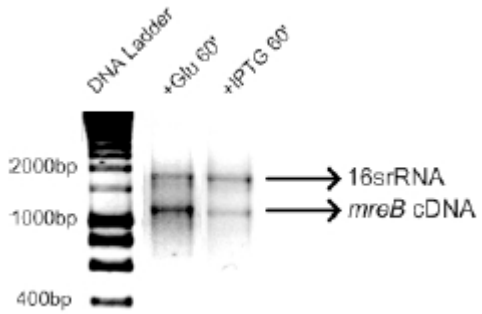

Fig. S4.Total RNA was extracted as previously (Santos et al., 1999). For cDNA synthesis Transcriptor First Strand cDNA Synthesis Kit (Roche) was used. Equal amounts of RNA were mixed with ramdom hexamer primer. Primer and RNA mixture were heated for 10min at 65°C for denaturation of RNA secondary structures. After addition of all components, samples were incubated for 10min at 25°C followed by 30min at 55°C. Inactivation of Transcriptor Reverse Transcriptase was done at 85°C for 5min. For PCR reaction 5ul of cDNA were used as template with Taq Polymerase (Roche) and primers RTMREB (5′-ACTTGTCCATTGACCTGGGTACTG-3′) and RTMREB2 (5′-GCCGCCGTGCATGTCGATCATTTC-3′). Equal amounts of sample were loaded in a 0.7% agarose gel. Quantification was done with ImageJ software and normalized by 16s rRNA levels obtained in same PCR reaction with primers 16S rRNA Forward (5′-AGAGTT TGATCCTGGCTCAG-3′) 16S rRNA Reverse (5′-ACGGCTACCTTGTTACGACTT-3′). The values provided in the text represent the amount of BolA detected divided by the amount of EF-Tu.

Chapter 4

Multiple target regulation by BolA: The impact on stress related small non-coding RNAs in *Escherichia coli*

This chapter contains data from:

Moreira RN, Dressaire C and Arraiano CM, (2011). Multiple target regulation by BolA: The impact on stress related small non-coding RNAs in *Escherichia coli*. In preparation

Abstract

The morphogene *bolA* induces a spherical shape when overexpressed and it has been established as a general stress response gene. The cell shape alteration caused by BolA involves several modifications on the outer membrane (OM) of the bacteria and cell division machinery. To study the global effect of BolA in *E. coli* mRNA transcription, we cloned its gene into a plasmid under the control of an arabinose inducible promoter and performed transcriptomic studies. Cell envelope integrity during stress and normal growth is controlled by the alternative sigma factor sigma E (σ^E), a widespread sigma factor in pathogenic and non-pathogenic bacteria. Of all sigma factors identified in the transcriptomic study, σ^E was the unique significantly affected. Moreover, this regulation was also reflected on the σ^E dependent sRNAs RybB and CyaR. Interestingly, the non-σ^E dependent CsrB, DsrA, RyhB, OmrA, OmrB and RydB sRNAs were also noticed to vary according to the BolA overexpression, being BolA able to up or down-regulate their expression. All BolA target sRNAs are somehow involved with cell division, OM proteins regulation or motility. In fact, currently, about a third of the *E. coli* sRNAs with known cellular functions are involved in the regulation of outer membranes proteins (OMP). The finding that BolA can repress or enhance the transcription of sRNAs in the cell indicates a broad impact on cellular features, such as morphology maintenance, cell envelope stress response and cell motility especially in stress conditions when *bolA* is induced.

Introduction

In stress conditions bacteria respond to environmental changes not only by morphology changes but also by global modifications in transcription. The morphogene *bolA* induces a spherical shape when overexpressed, and it has been established as a general stress response gene (Santos et al., 1999). The *bolA* gene expression is regulated in a complex manner both transcriptionally and post-transcriptionally (Freire et al., 2006a; Lange and Hengge-Aronis, 1991; Moreira et al., 2011; Santos et al., 2006). *bolA* increases biofilm formation (Vieira et al., 2004) and modulates cell permeability (Freire et al., 2006b). All this mechanisms can be related with the fact that BolA acts as a transcription factor of certain target genes related with cell division and cell morphology. For instance, it is known that BolA induces cell membrane D,D-carboxypeptidases (Aldea et al., 1988; Guinote et al., 2011; Santos et al., 2002) and represses the expression of the bacterial actin-like protein MreB (Freire et al., 2009a). Thus, BolA acts as a dual activity regulator which allows the activation of some genes while repressing others. This gives a regulatory versatility within a transcriptional network.

The cell shape alteration caused by BolA involves several modifications on the outer membrane (OM) of the bacteria. The OM of Gram-negative bacteria is crucial for viability of the cells; therefore stress responses have evolved in these organisms to maintain its integrity. These changes are often accomplished by the induction of alternative sigma factors, modulating RNA polymerase activity to specific promoters (Johansen et al., 2006). In *Escherichia coli* one of the key pathways involved in maintaining cell envelope integrity during stress and normal growth is controlled by the alternative sigma factor sigma E (σ^E). The σ^E response to envelope stress is well characterized (Ades, 2008). σ^E is sequestered in

an inactive form at the inner membrane under nonstress conditions. Perturbation of envelope homeostasis, caused by damage of the OM or the accumulation of unfolded outer membrane proteins (OMPs), triggers release of σ^E to the cytoplasm, where it directs RNA polymerase to transcribe the σ^E regulon (Gogol et al., 2011). σ^E promoter consensus-motifs have been identified in *E. coli* and *Salmonella* (Rhodius et al., 2006; Skovierova et al., 2006). These motifs are observed in the promoter regions of MicA and RybB small RNAs (sRNAs) (Vogel and Papenfort, 2006). The σ^E-dependent transcription of these two sRNA genes has been shown in *Salmonella* (Vogel and Papenfort, 2006) and *E. coli* (Johansen et al., 2006). Currently, about a third of the *E. coli* sRNAs with known cellular functions are involved in the regulation of OMPs. Bacterial sRNAs are widespread and functionally diverse regulators with a predominant relatively small size (Beisel and Storz, 2010). They have been extensively studied over the last years because of their high importance in the post-transcriptional regulation of bacterial gene expression. The most predominant class of sRNAs is dependent on the RNA chaperone Hfq to form base pairing interactions with target mRNAs. The interaction between the sRNAs and their target is responsible for changes in mRNA translation and stability influencing gene expression of the target mRNAs.

In this work we have used microarrays to identify potential targets for BolA in *E. coli* and show that when BolA is overexpressed in exponential phase, the *rpoE* mRNA is upregulated. All the other sigma factor did not show this increase in the level of their transcripts. Some sRNAs were included in the microarray and the presence of BolA affected eight of those sRNAs. The σ^E dependent genes *rybB* and *cyaR* were in this group. RybB is involved in the OM integrity and stress response (Johansen et al., 2006). CyaR, cyclic AMP-activated RNA) promotes decay of the *ompX* mRNA (Johansen et al., 2008). The variations

of the identified sRNAs were confirmed by Northern blot analysis and the possible direct regulation by BolA was studied.

Results

The global effect of BolA in exponential phase: BolA affects σ^E

BolA is known by the pleiotropic effects at cell wall and cell division level (Freire et al., 2009b; Guinote, 2011; Santos et al., 2002) and also to respond to a variety of stresses (Santos et al., 1999). In exponential phase, this gene is barely study since σ^{70} is the major sigma factor under these conditions (Aldea et al., 1989). To evaluate the physiologic impact of BolA in *E. coli* exponential phase of growth, the global overview of transcription of the cell was analyzed by microarray experiments. We have compared the *bolA* deletion strain in exponential phase with the same strain with *bolA* highly induced from an arabinose promoter. Different genes belonging to different categories were observed to vary in the conditions tested. Among those are stress related genes. Curiously enough, from the six sigma factors represented on the microarray, only one was noticeably upregulated. The *rpoE* RNA, coding the stress sigma factor E, was the identified target (Fig. 1A). Its levels were upregulated around threefold in the presence of BolA (Fig. 1B).

BolA influences multiple sRNAs involved in stress response

The OM proteins and cell envelope integrity are of major importance for cell survival in stress conditions. It was previously shown that two sRNAs belong to the σ^E regulon, the small non coding RNA RybB and MicA, having a major impact on the transcription of OM proteins (Johansen et al., 2006; Papenfort et al.,

2006; Thompson et al., 2007). Since BolA is a stress related protein with different effects on PBPs and also permeability on the cell (Freire et al., 2006b; Guinote, 2011; Santos et al., 2002), we were interested in the possible regulation of different

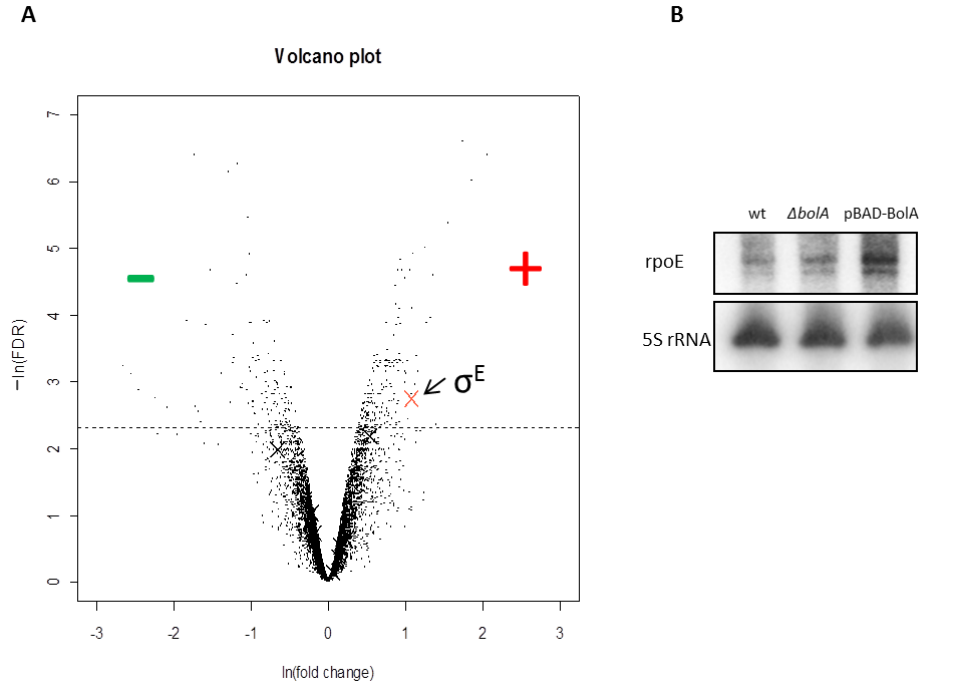

Fig. 1. (A) Volcano plot representation of the transcriptome results. Genes associated to a FDR lower than 10 % (represented by the horizontal scattered line) were considered significant. Each point corresponds to a gene, the black marks are all the σ-factors identified and the red cross is representing σ^E. The (+) and (-) represent the upregulated and downregulated areas in the plot respectively (B) Northern blot confirming the upregulation of σ^E mRNA.

sRNAs related with membrane stress response. Among 48 identified sRNAs in the microarrays study, 8 were considered as significantly up or downregulated (Fig. 2). Four transcripts were reduced > 1.75 fold, while four sRNAs showed ≥ 2 fold elevated levels (Fig. 3A). On average the upregulated RNAs exhibited a far higher degree of regulation. To confirm the transcriptomic data, we determined by Northern blot the RNA levels changes of the BolA-mediated downregulated

and upregulated for the selected targets (Fig. 3B). Of the eight selected genes, seven were confirmed to vary accordingly to the observed microarray data. Nevertheless, one of them was not downregulated but upregulated. For the two known sRNAs regulated by σ^E, we found that RybB is modulated in the cells ex-

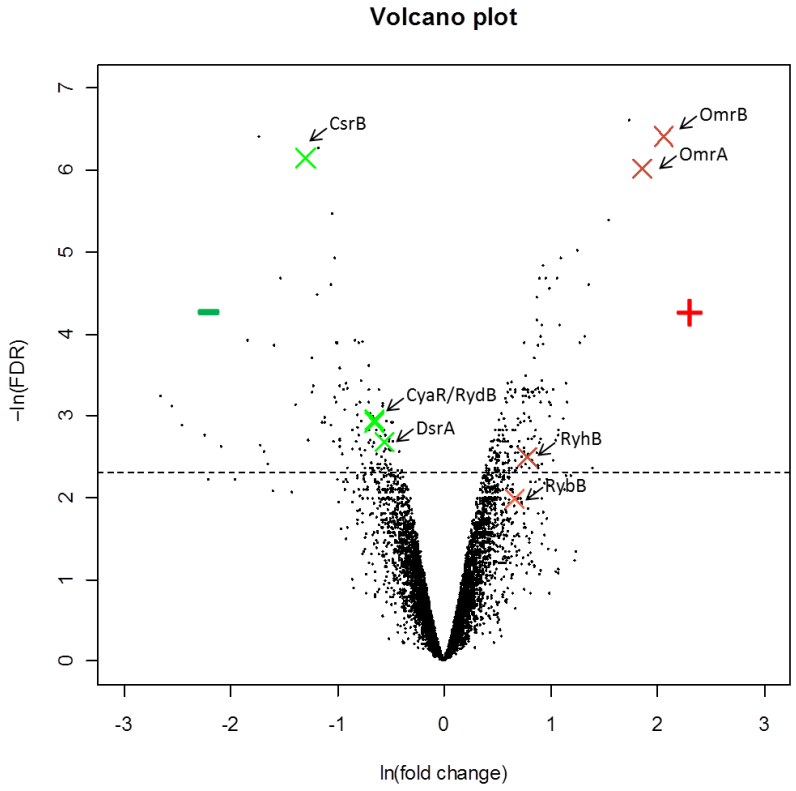

Fig. 2. Volcano plot showing the significantly downregulated or upregulated sRNAs. Genes associated to a FDR lower than 10 % (represented by the horizontal scattered line) were considered significant. The green and red marks are representing the sRNAs underexpressed and overexpressed respectively. The (+) and (-) represent the upregulated and downregulated areas in the plot respectively.

pressing BolA, being around twofold less abundant in the strain without BolA. However, for the thresholds used in the bioinformatic analyses, MicA was not detected to vary with BolA. CyaR represses OMP transcription when cells suffer

any kind of envelope stress (Johansen et al., 2008). Moreover, its levels are also influenced by σ^E even though it's not yet know if it's a direct regulation or not. According to these data, contrarily to what was observed in the microarray experiment, the variation observed in the Northern blots for CyaR is completely in agreement with expected, and CyaR goes up with more BolA, since σ^E is also upregulated in this condition. Of interest is also the pattern observed regarding the northern blot for RyhB. It seems that BolA is somehow necessary for the maintenance of this sRNA levels. In the *ΔbolA* strain, the amount RyhB mRNA is considerably lower than in the wt. This variation between wt and *ΔbolA* cells was not observed in the remaining sRNAs.

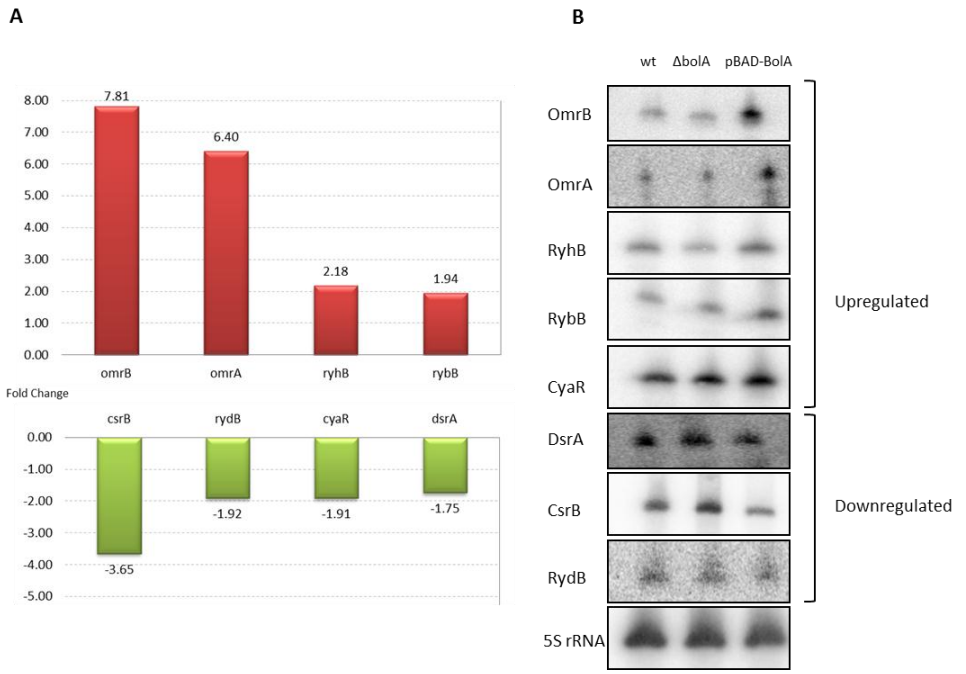

Fig. 3. (A) Graphical representation of the fold change of each studied sRNA. In red are the upregulated targets while in green are represented the downregulated. (B) Northern blot confirming the transcriptome mRNA variations.

BolA does not influence sRNAs decay rate and is probably acting at the level of transcription

The concentration of a given RNA in the cell it is not just a result of the rate of transcription but also the stability and alterations in mRNA decay. Both mRNA transcription and decay can control the levels of each protein in the cell. It was previously showm that BolA could directly or indirectly affect different mRNAs (Freire et al., 2009b; Guinote, 2011; Santos et al., 2002). In order to clarify in which way BolA is regulating sRNAs, mRNA stability was assessed by Northern blot and DNA-protein interactions were studied by Surface Plasmon Resonance (SPR). The decay rate of each sRNA was analyzed during 30min after 1h of BolA induction. Our preliminary results show no significant changes in the rate of the decay of the transcripts in the presence or absence of BolA (Fig. 5).

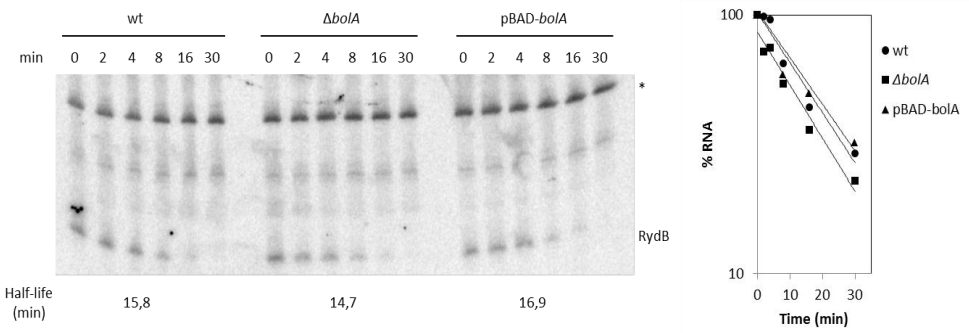

Fig. 4. Representative northern blot comparing the decay rate of the RydB RNA in the wild type, *ΔbolA* and pBAD-*bolA* strains in exponential phase. (*) Nonspecific band used as loading control. The same procedure was repeated for the other sRNAs involved in this study.

Since the stability was not altered, using SPR we tested the ability of this protein to bind to the promoter region of σ^E and each of the studied sRNA, to evaluate the possibility of BolA functioning as a transcription factor of these genes.

Preliminary data shows that the molecular interaction between BolA and σ^E promoter region was not significant. Accordingly, BolA is probably regulating the σ^E regulon sRNAs not indirectly via this stress sigma factor but by directly binding on their promoters. In fact, BolA has a slightly higher affinity to the promoters regions of five from the eight sRNAs that were found to be regulated in the tested conditions. However, further optimization of the technique and conditions used are necessary for the clarification of the results. Transcriptional fusions of the sRNAs regulatory regions with a *lacZ* gene are also undergoing to measure the impact of BolA in the rate of transcription.

Discussion

Small RNAs are very important in the post-transcriptional control of gene expression. It has been proposed that the expression of sRNAs in stationary phase may actually reflect transcriptional control by distinct stress regulons which are gradually activated upon the interruption of growth. Our new findings show that BolA, an *E. coli* transcription factor, is involved in the transcriptional regulation of sRNAs in exponential phase. BolA is regulated by σ^{70} and σ^S during exponential and stationary phase respectively (Aldea et al., 1989; Lange and Hengge-Aronis, 1991). However, when in stress conditions, the regulation of BolA is driven by the σ^S- regulated promoter even if cells are in exponential phase of growth (Santos et al., 1999). In order to better understand the global effect of BolA in this situation, we mimicked a stress condition during exponential phase by cloning BolA into a plasmid and under the control of an arabinose inducible promoter and performed transcriptomic studies. To put in evidence the effect of this transcriptional regulator, the *ΔbolA* strain overexpressing *bolA* was compared with a *ΔbolA* strain. Our results showed that a great variety of genes that are transcriptionally

modulated in a BolA-dependent manner, including a broad spectrum of genes involved in stress response.

Of all sigma factors identified in the transcriptomic study, the sigma factor σ^E was the unique sigma significantly affected. This sigma factor is widespread among a diverse set of pathogenic and non-pathogenic bacteria, and becomes activated when bacterial envelope homeostasis is disturbed (Rowley et al., 2006). The envelope stress response is a mechanism very important mainly in two different situations: when higher OMP production causes accumulation of misfolded OMPs in the periplasm and/or the envelope requires remodeling following damage by external stresses (Mecsas et al., 1993). When BolA was overexpressed in exponential phase, *rpoE* gene was observed to be upregulated around threefold. BolA overexpression causes morphologic changes in the cells (Freire et al., 2009b; Guinote, 2011; Santos et al., 2006) affecting different components of cell wall and cell division, changing morphology of the cells from rod to round shape. These changes are possibly influencing the homeostasis of the membrane and thus cause *rpoE* upregulation in response to the modifications. Moreover, this regulation reflected on the σ^E dependent sRNAs RybB and CyaR. Upon stress, RybB is upregulated in a σ^E-dependent manner limiting the OMP synthesis at the global scale (Johansen et al., 2006; Papenfort et al., 2006; Thompson et al., 2007). Even though for the threshold of 10% False Discovery Rate (FDR) used in the data processing the levels of RybB were not significantly overexpressed, we decided to study it due to its OMP regulation function. Furhtermore, CyaR is a member of Crp regulon that when overexpressed, represses OmpX levels (Papenfort et al., 2008). This small RNA is also involved in the regulation of *luxS*, a gene encoding a key enzyme in quorum sensing (De Lay and Gottesman, 2009), strengthening the possible link between BolA and motility

in bacteria/adhesion. Indeed, we have shown before that BolA is regulated by H-NS, a regulator of the flagella synthesis pathway (Moreira et al., 2011).

Not just σ^E-dependent sRNAs were identified as being regulated by BolA. CsrB, DsrA, RyhB, OmrA, OmrB and RydB were also noticed to vary according to the BolA overexpression. All these sRNAs are related with cell division, OM proteins regulation or motility, making the perfect connection with the described BolA functions. A regulatory network seems to be a reasonable idea for these targets and BolA. RydB, a novel non characterized sRNA, was observed to be reduced twofold by BolA. We searched for putative target for this sRNA and found that SdiA was a good match. Interestingly, SdiA is a protein described to affect several genes involved in cell division (Garcia-Lara et al., 1996; Sitnikov et al., 1996; Wang et al., 1991; Wei et al., 2001b; Yamamoto et al., 2001), a common feature with BolA cellular changes. However, this link between sRNA-target still needs to be better studied. CsrB is an antagonist of CsrA (Babitzke and Romeo, 2007), a protein that impede the translation of target mRNAs (Liu et al., 1995; Wei et al., 2001a). CsrA is known to positively affect the levels of the master flagella regulator *flhDC (Wei et al., 2001a)*. In this study, CsrB was observed to be downregulated by BolA, which subsequently would cause an increase of FlhDC (flagella). Flagella are important for motility, a feature required for the initial adhesion step of biofilm formation (Wood et al., 2006), which is promoted by BolA overexpression (Vieira et al., 2004).

bolA transcription is regulated by σ^S during stress conditions and at the same time negatively regulated by H-NS. DsrA is a negative regulator of H-NS and positively influences σ^S cellular levels (Sledjeski and Gottesman, 1995; Sledjeski et al., 1996). We observed a decrease of DsrA sRNA in our microarray study. A network of regulation between these four partners is an interesting hypothesis that would need to be addressed in a future study.

OmrA and OmrB, two sRNAs regulated by the OmpR transcriptional factor, were the most significant upregulated targets in the presence of BolA. Both are involved in OM proteins repression (Guillier and Gottesman, 2006), correlating with results obtained for σ^E-dependent sRNAs and their respective function in the membrane homeostasis.

This work shows that *bolA*-mediated cell morphology alterations and stress response are related not just by the genes modulated by this protein but also by a complex pathway that integrates σ^E and different stress related sRNAs. The finding that BolA can directly repress or enhance the transcription of sRNAs in the cell presents a broad impact on cellular features, such as morphology maintenance, cell envelope and cell motility especially in stress conditions when *bolA* is induced. Further studies will be necessary to provide more insights on these novel regulation pathways and how the different elements involved influence one another.

Material and Methods

Materials

Restriction enzymes, T4 DNA ligase, Pfu DNA polymerase and T4 Polynucleotide Kinase were purchased from Fermentas. DNaseI was purchased from Sigma. All the enzymes were used according to the supplier's instructions. Oligonucleotide primers used in this work were synthesized by STAB Vida, Portugal.

Bacterial Strains and Plasmids

The *E. coli* strains used were: DH5α (F' *fhuA2* Δ*(argF-lacZ)U169 phoA*

glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17a) for cloning experiments; JW5060 (*ΔbolA::kan*^{r}) (Baba et al., 2006); MG1655 (CSGS 6300) and CMA94 (MG1655 *ΔbolA::kan*^{r}), (Dressaire, C, et al, to be published). These strains were grown in Luria Broth medium (LB) at 37ºC, supplemented with 50μg/ml kanamycin, when required.

The *bolA* coding sequence was amplified by PCR using *E. coli* MG1655 chromosomal DNA and the primers bolANcoI and bolAKpnI. The amplified fragment was cut with NcoI and KpnI restriction enzymes and cloned into the pBAD/TorA vector previously cleaved with the same enzymes. The resulting plasmid (pCDA2) encoding BolA under an arabinose inducible promoter was used to transform *E. coli* CMA94 resulting in CMA95 (MG1655 *ΔbolA::kan*^{r}+pCDA2).

All constructions were confirmed by DNA sequencing at STAB Vida, Portugal.

RNA Extraction and northern blot analysis

Overnight cultures were diluted 1/100 in fresh LB medium and grown until mid-exponential phase. BolA was induced for 1h by addition of 0.14% arabinose. Culture samples were collected, mixed with 1 volume of stop solution [10mM Tris (pH 7.2), 25mM $NaNO_3$, 5mM $MgCl_2$, 500mg/ml chloramphenicol] and harvested by centrifugation (10min, 6000g, 4°C). For stability experiments, rifampicin (500mg/ml) and nalidixic acid (20mg/ml) were added to culture after the induction time. Culture aliquots were withdrawn at the time-points indicated in the respective figures. RNA was isolated using the phenol/chlorophorm extraction method, precipitated in ethanol, resuspended in water and quantified on a Nanodrop 1000 machine (NanoDrop Technologies).

For northern blot analysis, 15µg of total RNA was separated under denaturating conditions in a 8.3M urea/8% polyacrylamide gel in TBE buffer. Transfer of RNA onto Hybond-N+ membranes (GE Healthcare) was performed by electroblotting (1h 50min, 24V, 4°C) in TAE buffer. RNA was UV cross-linked to the membrane immediately after transfer. Membranes were then hybridized in RapidHyb Buffer (GE Healthcare) at 68°C for riboprobes and 42°C in the case of oligoprobes. After hybridization, membranes were washed as described (Viegas et al., 2007). Signals were visualized by PhosphorImaging (Storm Gel and Blot Imaging System, Amersham Bioscience) and analysed using the ImageQuant software (Molecular Dynamics).

Hybridization Probes

Riboprobe synthesis and oligoprobe labeling was performed as previously described (Viegas et al., 2007). PCR products used as template in the riboprobe synthesis were obtained using the following primer pairs: rnm030/rnm031 for CsrB, rnm032/rnm033 for CyaR, rnm034/rnm035 for RyhB, rnm038/rnm039 for RybB and rnm058/rnm059 for *rpoE*. The DNA probes for were generated using the primers rnm036 for OmrA, rnm037 for OmrB, rnm040 for DsrA, rnm041 for RydB and 16sR labeled at 5′ end with [γ-32P]ATP using T4 polynucleotide kinase (Fermentas).

Microarrays

RNA quality control was evaluated with a BioAnalyzer (Agilent Technology). Processing of extracted RNA, cDNA labelling, hybridization and slide-scanning procedures were performed according to manufacturer's instructions found in the 'Affymetrix Gene Expression Technical Manual'

(http://www.affymetrix.com). Hybridization, scanning and detection procedures were done at the Genomics Unit of the Instituto Gulbenkian de Ciência (Portugal).

Affymetrix GeneChip provides 22 independent measurements for each genome target. Those measurements come from 11 probe pairs composed of single 25-mer perfect match (PM) oligo and its corresponding 25-mer mismatch (MM) oligo. The mismatch oligo is identical to the perfect match with the exception of a single nucleotide mismatch located at the central (13th) position of the oligo sequence. The single probe set intensity value for a given target is obtained through the summary of these 22 independent measurements.

Microarrays data analysis

Analysis of the generated Affymetrix CEL files was performed using R free statistical software (http://cran.r-project.org/) and its associated tool for high-throughput genomic data, Bioconductor (http://www.bioconductor.org/). The reliability of the data set, before and after normalization, was estimated through its statistical exploration (ie. box-plots to assay median stability and variability among repetitions; histograms of the log-transformed value to visualize the Gaussian distribution; multiple scatter-plots and clustering to check the reproducibility of the repetitions and clustering; graphs not shown). For each strain, the summarized probe set intensities were calculated using the Robust MultiArray Averaging (RMA) method, which provides high sensitivity and specificity in detection of differential expression (Bolstad et al., 2003; Irizarry et al., 2003). RMA includes global background adjustment, across-array quantile normalization and performs median polish separately for each probe set to give log-transformed PM values. The multiple testing issue was furthermore taken

into account through the calculation of the False Discovery Rate (FDR) according to Benjamini-Hochberg method (Benjamini et al., 2001). Genes displaying CMA95 (overexpressing BolA) vs. CMA94 (*ΔbolA*) ratio associated to a FDR lower than 10 % were considered as differentially regulated. It was checked that the t-statistic p-value associated was lower than 5 % with a mean of value of 0.11 and 1.01 % for the comparison 1h after BolA induction.

sRNA target prediction

In silico prediction of putative targets in the *Escherichia coli* genome for RydB sRNA was performed using the TargetRNA software (Tjaden, 2008; Tjaden et al., 2006) bioinformatic tool.

Acknowledgments

We thank Andreia Aires for technical assistance. Fundação para a Ciência e Tecnologia gave financial support to R. Moreira (Doctoral fellowship), C. Dressaire (Postdoctoral fellowship) and R. Matos (Doctoral fellowship). This work was supported by Fundação para a Ciência e Tecnologia (FCT) in Portugal.

References

Ades, S.E. 2008. Regulation by destruction: design of the sigmaE envelope stress response. Curr Opin Microbiol. 11:535-540.

Aldea, M., T. Garrido, C. Hernandez-Chico, M. Vicente, and S.R. Kushner. 1989. Induction of a growth-phase-dependent promoter triggers transcription of bolA, an Escherichia coli morphogene. EMBO J. 8:3923-3931.

Aldea, M., C. Hernandez-Chico, A.G. de la Campa, S.R. Kushner, and M. Vicente. 1988. Identification, cloning, and expression of bolA, an ftsZ-dependent morphogene of Escherichia coli. J Bacteriol. 170:5169-5176.

Baba, T., T. Ara, M. Hasegawa, Y. Takai, Y. Okumura, M. Baba, K.A. Datsenko, M. Tomita, B.L. Wanner, and H. Mori. 2006. Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. Molecular Systems Biology. 2.

Babitzke, P., and T. Romeo. 2007. CsrB sRNA family: sequestration of RNA-binding regulatory proteins. Curr Opin Microbiol. 10:156-163.

Beisel, C.L., and G. Storz. 2010. Base pairing small RNAs and their roles in global regulatory networks. FEMS Microbiol Rev. 34:866-882.

Benjamini, Y., D. Drai, G. Elmer, N. Kafkafi, and I. Golani. 2001. Controlling the false discovery rate in behavior genetics research. Behav Brain Res. 125:279-284.

Bolstad, B.M., R.A. Irizarry, M. Astrand, and T.P. Speed. 2003. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics. 19:185-193.

De Lay, N., and S. Gottesman. 2009. The Crp-activated small noncoding regulatory RNA CyaR (RyeE) links nutritional status to group behavior. J Bacteriol. 191:461-476.

Freire, P., J.D. Amaral, J.M. Santos, and C.M. Arraiano. 2006a. Adaptation to carbon starvation: RNase III ensures normal expression levels of bolA1p mRNA and sigma(S). Biochimie. 88:341-346.

Freire, P., R.N. Moreira, and C.M. Arraiano. 2009a. BolA inhibits cell elongation and regulates MreB expression levels. J Mol Biol. 385:1345-1351.

Freire, P., R. Neves Moreira, and C.M. Arraiano. 2009b. BolA inhibits cell elongation and regulates MreB expression levels. J Mol Biol. 385:1345-1351.

Freire, P., H.L. Vieira, A.R. Furtado, M.A. de Pedro, and C.M. Arraiano. 2006b. Effect of the morphogene bolA on the permeability of the Escherichia coli outer membrane. FEMS Microbiol Lett. 260:106-111.

Garcia-Lara, J., L.H. Shang, and L.I. Rothfield. 1996. An extracellular factor regulates expression of sdiA, a transcriptional activator of cell division genes in Escherichia coli. J Bacteriol. 178:2742-2748.

Gogol, E.B., V.A. Rhodius, K. Papenfort, J. Vogel, and C.A. Gross. 2011. Small RNAs endow a transcriptional activator with essential repressor functions for single-tier control of a global stress regulon. Proc Natl Acad Sci U S A. 108:12875-12880.

Guillier, M., and S. Gottesman. 2006. Remodelling of the Escherichia coli outer membrane by two small regulatory RNAs. Mol Microbiol. 59:231-247.

Guinote, I.B., R.G. Matos, P. Freire, and C.M. Arraiano. 2011. BolA affects cell growth, and binds to the promoters of penicillin-binding proteins 5 and 6 and regulates their expression. J Microbiol Biotechnol. 21:243-251.

Guinote, I.B., Rute Gonçalves Matos, Patrick Freire, and Cecília Maria Arraiano. 2011. BolA Affects Cell Growth, and Binds to the Promoters of Penicillin-Binding Proteins 5 and 6 and Regulates Their Expression. Journal of Microbiology and Biotechnology. in press.

Irizarry, R.A., B. Hobbs, F. Collin, Y.D. Beazer-Barclay, K.J. Antonellis, U. Scherf, and T.P. Speed. 2003. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics. 4:249-264.

Johansen, J., M. Eriksen, B. Kallipolitis, and P. Valentin-Hansen. 2008. Down-regulation of outer membrane proteins by noncoding RNAs: unraveling the cAMP-CRP- and sigmaE-dependent CyaR-ompX regulatory case. J Mol Biol. 383:1-9.

Johansen, J., A.A. Rasmussen, M. Overgaard, and P. Valentin-Hansen. 2006. Conserved small non-coding RNAs that belong to the sigmaE regulon: role in down-regulation of outer membrane proteins. J Mol Biol. 364:1-8.

Lange, R., and R. Hengge-Aronis. 1991. Growth phase-regulated expression of bolA and morphology of stationary-phase Escherichia coli cells are controlled by the novel sigma factor sigma S. J Bacteriol. 173:4474-4481.

Liu, M.Y., H. Yang, and T. Romeo. 1995. The product of the pleiotropic Escherichia coli gene csrA modulates glycogen biosynthesis via effects on mRNA stability. J Bacteriol. 177:2663-2672.

Mecsas, J., P.E. Rouviere, J.W. Erickson, T.J. Donohue, and C.A. Gross. 1993. The activity of sigma E, an Escherichia coli heat-inducible sigma-factor, is modulated by expression of outer membrane proteins. Genes Dev. 7:2618-2628.

Moreira, R.N., C. Dressaire, S. Domingues, and C.M. Arraiano. 2011. A new target for an old regulator: H-NS represses transcription of bolA morphogene by direct binding to both promoters. Biochem Biophys Res Commun. 411:50-55.

Papenfort, K., V. Pfeiffer, S. Lucchini, A. Sonawane, J.C. Hinton, and J. Vogel. 2008. Systematic deletion of Salmonella small RNA genes identifies CyaR, a conserved CRP-dependent riboregulator of OmpX synthesis. Mol Microbiol. 68:890-906.

Papenfort, K., V. Pfeiffer, F. Mika, S. Lucchini, J.C. Hinton, and J. Vogel. 2006. SigmaE-dependent small RNAs of Salmonella respond to membrane stress by accelerating global omp mRNA decay. Mol Microbiol. 62:1674-1688.

Rhodius, V.A., W.C. Suh, G. Nonaka, J. West, and C.A. Gross. 2006. Conserved and variable functions of the sigmaE stress response in related genomes. PLoS Biol. 4:e2.

Rowley, G., M. Spector, J. Kormanec, and M. Roberts. 2006. Pushing the envelope: extracytoplasmic stress responses in bacterial pathogens. Nat Rev Microbiol. 4:383-394.

Santos, J.M., P. Freire, F.S. Mesquita, F. Mika, R. Hengge, and C.M. Arraiano. 2006. Poly(A)-polymerase I links transcription with mRNA degradation via sigmaS proteolysis. Mol Microbiol. 60:177-188.

Santos, J.M., P. Freire, M. Vicente, and C.M. Arraiano. 1999. The stationary-phase morphogene bolA from Escherichia coli is induced by stress during early stages of growth. Mol Microbiol. 32:789-798.

Santos, J.M., M. Lobo, A.P. Matos, M.A. De Pedro, and C.M. Arraiano. 2002. The gene bolA regulates dacA (PBP5), dacC (PBP6) and ampC (AmpC), promoting normal morphology in Escherichia coli. Mol Microbiol. 45:1729-1740.

Sitnikov, D.M., J.B. Schineller, and T.O. Baldwin. 1996. Control of cell division in Escherichia coli: regulation of transcription of ftsQA involves both rpoS and SdiA-mediated autoinduction. Proc Natl Acad Sci U S A. 93:336-341.

Skovierova, H., G. Rowley, B. Rezuchova, D. Homerova, C. Lewis, M. Roberts, and J. Kormanec. 2006. Identification of the sigmaE regulon of Salmonella enterica serovar Typhimurium. Microbiology. 152:1347-1359.

Sledjeski, D., and S. Gottesman. 1995. A small RNA acts as an antisilencer of the H-NS-silenced rcsA gene of Escherichia coli. Proc Natl Acad Sci U S A. 92:2003-2007.

Sledjeski, D.D., A. Gupta, and S. Gottesman. 1996. The small RNA, DsrA, is essential for the low temperature expression of RpoS during exponential growth in Escherichia coli. EMBO J. 15:3993-4000.

Thompson, K.M., V.A. Rhodius, and S. Gottesman. 2007. SigmaE regulates and is regulated by a small RNA in Escherichia coli. J Bacteriol. 189:4243-4256.

Tjaden, B. 2008. TargetRNA: a tool for predicting targets of small RNA action in bacteria. Nucleic Acids Res. 36:W109-113.

Tjaden, B., S.S. Goodwin, J.A. Opdyke, M. Guillier, D.X. Fu, S. Gottesman, and G. Storz. 2006. Target prediction for small, noncoding RNAs in bacteria. Nucleic Acids Res. 34:2791-2802.

Viegas, S.C., V. Pfeiffer, A. Sittka, I.J. Silva, J. Vogel, and C.M. Arraiano. 2007. Characterization of the role of ribonucleases in Salmonella small RNA decay. Nucleic Acids Res. 35:7651-7664.

Vieira, H.L., P. Freire, and C.M. Arraiano. 2004. Effect of Escherichia coli morphogene bolA on biofilms. Appl Environ Microbiol. 70:5682-5684.

Vogel, J., and K. Papenfort. 2006. Small non-coding RNAs and the bacterial outer membrane. Curr Opin Microbiol. 9:605-611.

Wang, X.D., P.A. de Boer, and L.I. Rothfield. 1991. A factor that positively regulates cell division by activating transcription of the major cluster of essential cell division genes of Escherichia coli. EMBO J. 10:3363-3372.

Wei, B.L., A.M. Brun-Zinkernagel, J.W. Simecka, B.M. Pruss, P. Babitzke, and T. Romeo. 2001a. Positive regulation of motility and flhDC expression by the RNA-binding protein CsrA of Escherichia coli. Mol Microbiol. 40:245-256.

Wei, Y., J.M. Lee, D.R. Smulski, and R.A. LaRossa. 2001b. Global impact of sdiA amplification revealed by comprehensive gene expression profiling of Escherichia coli. J Bacteriol. 183:2265-2272.

Wood, T.K., A.F. Gonzalez Barrios, M. Herzberg, and J. Lee. 2006. Motility influences biofilm architecture in Escherichia coli. Appl Microbiol Biotechnol. 72:361-367.

Yamamoto, K., K. Yata, N. Fujita, and A. Ishihama. 2001. Novel mode of transcription regulation by SdiA, an Escherichia coli homologue of the quorum-sensing regulator. Mol Microbiol. 41:1187-1198.

Supplementary Data

Table 1. Oligonucleotides used in this work

Oligonucleotides	Sequence
bolANcoI	5′-CATGCCATGGATGACATCTCAGCGTTGTCG-3′
bolAKpnI	5′-GCAAGGTACCAGACAAAACAAAACGGCCCG-3′
rnm030	5′-GTAATACGACTCACTATAGGTTCGTTTCGCAGCATTCCAG-3′
rnm031	5′-GCGTTAAAGGACACCTCCAGG-3′
rnm032	5′-GTAATACGACTCACTATAGGCTAAGGAGGTGGTTCCTGG-3′
rnm033	5′-GCTGAAAAACATAACCC-3′
rnm034	5′-GTAATACGACTCACTATAGAAAAGCCAGCACCCGGCTG-3′
rnm035	5′-GCGATCAGGAAGACCCTCG-3′
rnm036	5′-GAGACAGGGTACGAAGAGCGTACCGAATAATCTCACC-3′
rnm037	5′-GTGTAATTCATGTGCTCAACCCGAAGTTGACTTCACC-3′
rnm038	5′-TAATACGACTCACTATAGGAACAAAAAACCCATCAACCTTGAACCG-3′
rnm039	5'-ACTGCTTTTCTTTGATGTCCC-3'
rnm040	5′-AAATCCCGACCCTGAGGGGGTCGGGATGAAACTTGCTTAAGC-3′
rnm041	5′-CTACCCCATCCGGCGCTTATCTCCGGCACTCTCAGTGGCTTAG-3′
rnm058	5′-TGAGCGAGCAGTTAACGGAC-3′
rnm059	5′-TAATACGACTCACTATAGGAGGCCATCCAGCTCCCGCAAG-3′

Chapter 5

Criss-cross regulation of RNase R and SmpB in *Streptococcus pneumoniae*

This chapter contains data of the submitted article:

Moreira RN[a,*], Domingues SD[a,*], Viegas SC[a], Amblar M[b] and Arraiano, CM[a], (2011). Criss-cross regulation of RNase R and SmpB in *Streptococcus pneumoniae*. Submitted in J. Mol. Biol.

[a]Instituto de Tecnologia Química e Biológica / Universidade Nova de Lisboa, Apartado 127, 2781-901 Oeiras, Portugal.

[b]Centro Nacional de Microbiología, Instituto de Salud Carlos III. Ctra. Majadahonda-Pozuelo Km 2.200. Majadahonda, 28220 Madrid, Spain, and CIBER Enfermedades Respiratorias

*Contributed equally

Abstract

Ribonuclease R (RNase R) is an exoribonuclease that recognizes and degrades a wide range of RNA molecules. It is a stress-induced protein shown to be important for the establishment of virulence in some pathogenic bacteria. This enzyme, together with the SmpB-tmRNA system is also involved in the trans-translation process, a translational quality control system that resolves challenges associated with stalled ribosomes on defective mRNAs. Trans-translation has also been associated with deficiencies in stress-response mechanisms and pathogenicity. In this work we study the expression of RNase R in the human pathogen *Streptococcus pneumoniae* and analyze the enzyme's involvement with the main components of the trans-translation machinery (SmpB and tmRNA/SsrA). We show that RNase R is induced after a 37 °C to 15 °C temperature downshift and that its levels are dependent on the trans-translation mediator SmpB. Transcriptional analysis of the *S. pneumoniae rnr* gene reveal that it is co-transcribed with the flanking genes, *secG* and *smpB*. Transcription of the operon is driven from a single promoter mapped upstream of *secG*. The association of *secG*, *rnr* and *smpB* seems a common feature of Gram positive bacteria, and the biological significance of this gene cluster is further discussed. This study unravels an additional contribution of RNase R to the trans-translation system, since the levels of SmpB are also shown to be under the control of this exoribonuclease. These proteins are therefore mutually dependent and cross-regulated.

Introduction

The ability of bacteria to sense and adapt to environmental changes is critical to survival. Under stress conditions, prokaryotic cells must rapidly alter their gene expression to deal with a changing environment. RNA molecules provide the dynamic link between DNA-encoded information and protein synthesis. A rapid genetic response to a changing environment involves not only transcriptional but also post-transcriptional regulation (Arraiano et al., 2010; Arraiano and Maquat, 2003). The labile nature of RNA is critical as it allows a rapid adjustment of proteins levels. Therefore, mRNA decay is of prime importance for controlling gene expression.

RNase R is a processive 3′-5′ exoribonuclease that belongs to the RNase II family of enzymes (Andrade et al., 2009; Cheng and Deutscher, 2002; Grossman and van Hoof, 2006; Zuo and Deutscher, 2001). Orthologues have been found in most sequenced genomes (Condon and Putzer, 2002) and have been implicated in the processing and degradation of different types of RNA, such as tRNA, rRNA, mRNA and small RNAs (sRNAs) (Andrade et al., 2006; Andrade et al., 2009; Cairrao et al., 2003; Cheng and Deutscher, 2003; Fonseca et al., 2008; Lalonde et al., 2007; Oussenko et al., 2005). RNase R is the only exoribonuclease able to degrade highly structured RNA molecules and therefore, it is particularly important in the removal of RNA fragments with extensive secondary structures (Cheng and Deutscher, 2005). Such ability of RNase R is probably on the basis of its marked increase during cold-shock, a condition which thermodynamically favors the formation of highly structured RNA molecules. In fact, *E. coli* RNase R seems to be a general stress-induced protein whose levels are not only upregulated under cold-shock , but also upon entry into stationary phase and in response to heat shock (Andrade et al., 2006; Cairrao et al., 2003; Chen and Deutscher, 2005). Stress resistance and virulence are intimately related since

many pathogenic bacteria are challenged with very harsh conditions during the process of infection. Not surprisingly, RNase R has been implicated in the establishment of virulence in a growing number of pathogens. These include *Aeromonas hydrophyla*, *Shigella flexneri*, enteroinvasive *Escherichia coli*, and *Helicobacter pylori* (Cheng et al., 1998; Erova et al., 2008; Tobe et al., 1992; Tsao et al., 2009). The enzyme has also been involved the quality control of defective tRNAs and rRNA molecules (Campos-Guillen et al., 2010; Cheng and Deutscher, 2003). Furthermore, *E. coli* RNase R was shown to participate in the maturation of the transfer-messenger RNA (tmRNA, also called SsrA) (Cairrao et al., 2003), an important small RNA involved in the protein quality control. In *Pseudomonas syringae* and *Caulobacter crescentus*, degradation of tmRNA was also shown to be dependent on RNase R (Hong et al., 2005; Purusharth et al., 2007). tmRNA together with SmpB are the main components of the trans-translation system, an elegant surveillance pathway that targets deficient proteins and mRNAs for degradation while rescuing stalled ribosomes (for a revision see references (Keiler, 2008; Richards et al., 2008)). Efficiently released ribosomes can participate in new productive translation events, and elimination of aberrant mRNAs and proteins provides recycled nutrients to the cell. *E. coli* RNase R was further shown to be physically associated with the tmRNA/SmpB system (Karzai and Sauer, 2001) and SmpB was demonstrated to regulate RNase R stability in a tmRNA-dependent manner (Liang and Deutscher, 2010). Moreover, the enzyme is the key exoribonuclease involved in the degradation of the faulty mRNAs released after ribosome rescue (Ge et al., 2010; Richards et al., 2006). The rapid removal of these defective messages is of utmost importance in the prevention of future stalling events. Trans-translation also allows bacteria to efficiently respond to a variety of stresses, is required for the viability of many pathogenic bacteria and is necessary for virulence in some pathogens (reviewed by (Keiler, 2008; Richards et al., 2008)).

In previous studies we have biochemically characterized RNase R from *Streptococcus pneumoniae* (Domingues et al., 2009), an important human pathogen that causes bacterial pneumonia, septicaemia, meningitis and otitis media. Interestingly, analysis of *S. pneumoniae* genome revealed that the coding sequence of SmpB is located immediately downstream of the gene encoding RNase R (*rnr*) (Fig. 1). Together, these observations prompted us to study RNase R expression in this bacterium and to analyze the involvement of this exoribonuclease with the trans-translation machinery of *S. pneumoniae*. In this report we show that cold-shock stress induces both *rnr* mRNA and RNase R protein levels. Besides temperature, we demonstrate that RNase R levels are also modulated by SmpB. Furthermore, we uncover an additional implication of RNase R with the trans-translation machinery, showing for the first time that RNase R is involved in the control of SmpB levels. The pneumococcal *rnr* gene is co-transcribed in operon with *smpB* and *secG*. The possible meaning of this clustered genomic location is discussed.

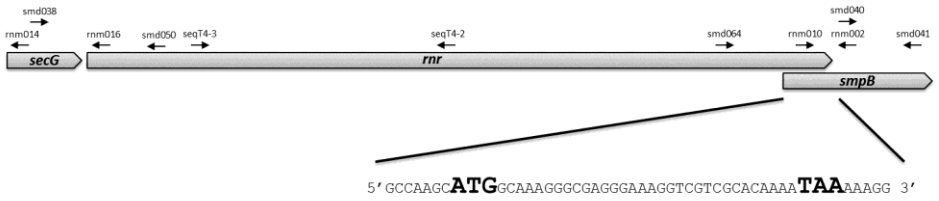

Fig. 1 - Genomic organization of the *rnr* region in *S. pneumoniae*. A schematic representation of *rnr* and its flanking genes – *secG* and *smpB* - is shown. To facilitate analysis of the results all primers used in RT-PCR and primer extension experiments are represented by arrows indicating their approximate location and orientation (sense/antisense). The overlapping region between *rnr* and *smpB* is depicted. In the sequence, the *smpB* and *rnr* respective start and stop codons are shown in bold.

Results

RNase R Levels are Regulated by Temperature and Modulated by SmpB

RNase R is the only hydrolytic exoribonuclease described in *S. pneumoniae* (Domingues et al., 2009). This enzyme has previously been biochemically characterized (Domingues et al., 2009) but its role in the cell is still unknown. In *E. coli*, *C. crescentus* and *P. syringae* RNase R was shown to have a role in the quality control of protein synthesis through its involvement with the trans-translation system, together with SmpB protein and tmRNA (Cairrao et al., 2003; Hong et al., 2005; Purusharth et al., 2007; Richards et al., 2006). Interestingly, analysis of the *rnr* genomic region of *S. pneumoniae* revealed that the coding sequence of SmpB is located immediately downstream of the RNase R coding gene. Throughout the infection process, this human pathogen is exposed to numerous stress conditions, namely temperature changes. RNase R was previously described to be modulated in response to different stress situations, and after cold-shock treatment RNase R levels are markedly increased (Andrade et al., 2006; Cairrao et al., 2003; Chen and Deutscher, 2005). Altogether these observations encouraged us to characterize *S. pneumoniae* RNase R expression and to analyze the enzyme´s involvement with the trans-translation machinery in this bacterium.

To study the expression of RNase R, total protein extracts obtained under physiological temperature and cold-shock were analysed by Western immunoblotting. We have purified RNase R from *S. pneumoniae* and the purified protein was used to raise specific polyclonal antibodies. Western blot experiments performed with these antibodies showed that after a downshift from 37 °C to 15

°C the protein levels considerably increased (Fig. 2). RNase R was not detected in an RNase R deficient mutant. This result shows that the expression of the pneu-

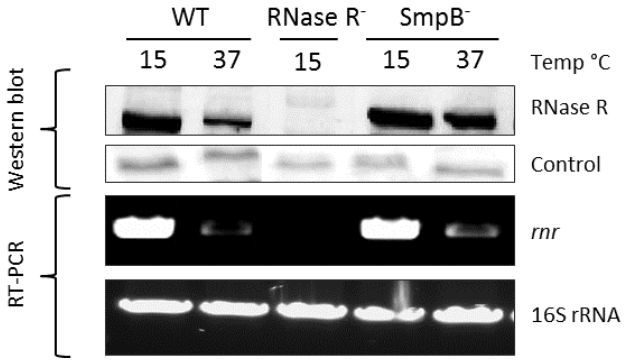

Fig. 2 - Pneumococcal RNase R (~92 kDa) and its respective mRNA are more abundant under cold-shock. Western blot and RT-PCR analysis of protein and RNA samples extracted from wild-type and mutant strains as indicated on top of each lane. Details of experimental procedures are described in 'Materials and Methods' section. (Upper panel) Analysis of RNase R expression by Western immunoblotting. RNase R levels were compared in the wild-type (WT) and in the SmpB- mutant at different temperatures (15 °C and 37 °C). 20 μg of each protein sample were separated in a 7 % tricine-SDS-polyacrylamide gel and blotted to a nitrocellulose membrane. RNase R was detected using specific antibodies. An RNase R- mutant strain was used as a negative control. A non-specific band (Control) detected with the same antibodies was used as loading control. (Lower panel) Analysis of *rnr* mRNA levels by RT-PCR. RT–PCR experiments were carried out with primers specific for *rnr* using 100 ng of total RNA extracted from the wild type (WT) and SmpB- mutant at different temperatures (15 °C, 37 °C). The RNase R- mutant derivative was used as a negative control. RT-PCR with primers specific for 16S rRNA shows that there were not significant variations in the amount of RNA used in each sample.

-mococcal RNase R is modulated by temperature and is highly increased under cold-shock. In order to determine whether the induction of RNase R could be related with a higher level of the *rnr* transcript in these conditions, the variation of the *rnr* mRNA levels was studied by RT-PCR. Similarly to the Western blot results, a strong increase in the amount of the *rnr* transcript was observed under cold-shock (Fig. 2). Therefore, the higher levels of RNase R at 15 °C could probably be a consequence of the strong increase of the respective mRNA.

It has been recently shown that the stability of *E. coli* RNase R is reduced by SmpB and tmRNA (Liang and Deutscher, 2010). To see if this also happened with the pneumococcal RNase R, comparative Western blot analysis was performed in the presence or absence of SmpB. For this purpose we have constructed an isogenic mutant lacking *smpB* (SmpB-) and followed the expression of RNase R in the wild type and the mutant strain at 15 °C and 37 °C. In the presence of SmpB the levels of RNase R are strongly increased at 15 °C. By contrast, when SmpB is absent RNase R levels remained high at 37 °C (Fig. 2). Furthermore, the levels of the *rnr* transcript in the SmpB- mutant resemble those of the wild-type strain, whether at 15 °C or at 37 °C (Fig. 2). This suggests that the difference in the amount of protein observed in the absence of SmpB at 37 °C was not linked with the *rnr* mRNA levels.

This result indicates that similarly to what was observed in *E. coli* (Liang and Deutscher, 2010) in *S. pneumoniae* SmpB may be one important factor in controlling the stability of RNase R. Nonetheless, the dramatic increase in the *rnr* mRNA levels under cold-shock may certainly account for the final levels of RNase R in the cell, as it was observed in *E. coli*.

RNase R Transcriptional Unit: secG, rnr and smpB are Co-transcribed in Operon

The cooperation of RNase R and SmpB in important cellular functions, together with the proximal location of their respective coding sequences in the genome of *S. pneumoniae*, led us to further characterize the expression of these two genes. The fact that the *rnr* gene is located upstream and partially overlaps with smpB (see Fig. 1) indicates that these genes may be co-transcribed as part of an operon. Furthermore, by bioinformatics analysis no promoter could be identified in the region upstream of *smpB*, suggesting that the expression of this gene is coupled with that of *rnr*. To study the *rnr* transcript and its transcriptional unit, RT-PCR experiments were carried out using primers smd064 (annealing specifically with *rnr*) and smd041 (annealing specifically *smpB*) (see localization of primers in Fig 1). As shown in Figure 3 (Lane 1), a fragment that results from the amplification of a transcript containing both *rnr* and *smpB* could be observed, indicating that *rnr* is co-transcribed with *smpB*. To confirm this hypothesis, primer

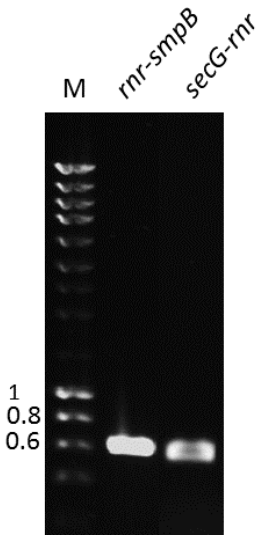

Fig. 3 - *rnr* is co-expressed with *secG* and *smpB*. *secG-rnr* and *rnr-smpB* transcripts were detected by RT-PCR. Molecular weight marker is shown on the left. *rnr-smpB* – RT-PCR was performed with 100 ng of total RNA extracted from the wild type strain at 15 °C. One of the primers was specific for rnr and the other for *smpB*. *secG-rnr* - RT-PCR was carried out using a *secG* specific primer and an *rnr* specific primer on 200 ng of total RNA extracted from the wild type strain at 15 °C. In any case, parallel RT-PCR reactions run in the absence of reverse transcriptase yielded no product.

extension assays using a primer specific for the *smpB* 5'-end region (rnm002 – see Fig 1 and Table S1) were performed. As shown in Figure 4a, four different fragments were extended from this primer. Analysis of the sequence revealed that

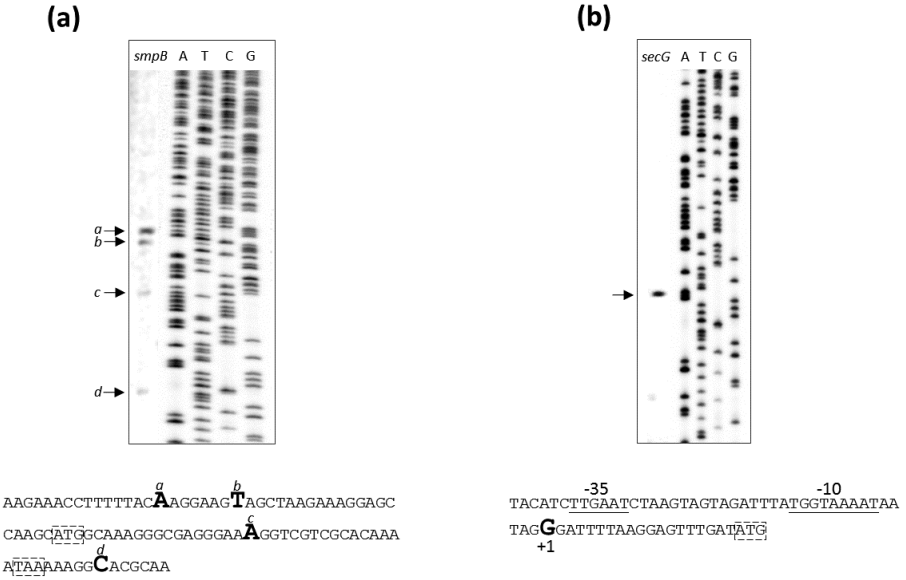

Fig. 4 - Primer extension analysis of the rnr genomic region. ATCG lanes are sequencing ladders obtained with M13 DNA and a specific radiolabeled primer. (a) Primer extension was carried out with 5 μg of total RNA extracted from the RNase R- strain at 15 ºC using a 5'-end-labeled primer specific for the 5'region of smpB (rnm002). The arrows indicate the fragments (a, b, c and d) extended from this primer. Sequence of the region that comprises the 3'end of rnr and the 5'end of smpB is indicated on the bottom. The nucleotides corresponding to the 5'-end of the extended fragments (a, b, c and d) are highlighted in bold. The ATG of smpB and the stop codon of rnr (TAA) are indicated by a dashed box. (b) Primer extension using 5 μg of total RNA extracted from the wild type at 15 ºC and a 5'-end-labeled primer specific for the 5'region of secG (rnm014). The arrow indicates the fragment extended with this primer. Sequence of the region upstream of secG is indicated on the bottom. The nucleotide corresponding to +1, as determined by the size of the extended fragment, is shown in bold. The -35 and -10 boxes are underlined, and the ATG start codon of secG is indicated by a dashed box.

the 5′-end of each fragment likely corresponds to a different processing site in the overlapping region between *rnr* and *smpB*. The localization of these processing sites suggests that the transcript containing both *rnr* and *smpB* is further processed to yield either single *rnr* or *smpB* mRNA (Fig. 4a). The different intensity of the fragments indicates that the *rnr-smpB* transcript is more frequently processed upstream the 3′-end of *rnr* (fragments a and b), giving rise to truncated *rnr* transcripts, which are most probably rapidly degraded by the RNA degradation machinery of the cell.

We proceeded to identify the promoters implicated in the transcription of this operon. Even though bioinformatics analysis indicated a putative promoter with satisfactory score immediately upstream of *rnr*, we could not identify any primer extension product resulting from primer hybridization at the 5′-end of *rnr* mRNA (data not shown). Upstream of *rnr* lays a small ORF that encodes a protein with homology to SecG, an auxiliary protein in the Sec-dependent protein export pathway. Since a putative promoter upstream this ORF was also identified *in silico*, we raised the hypothesis that transcription of *rnr* and *smpB* could be coupled with that of *secG* and would be directed from this promoter. To test this hypothesis we performed RT-PCR experiments using a primer specific for *secG* (smd038) together with an *rnr* specific primer (smd050) (see Fig. 1 for primers localization). An amplification product corresponding to a transcript that included *secG* and *rnr* was successfully detected, clearly showing that these two genes are also present in the same transcriptional unit (Fig. 3 – Lane 2). Thereby, a single transcriptional unit containing the three genes, *secG*, *rnr* and *smpB*, probably exists in the cell. In order to determine if the putative promoter identified upstream of *secG* could be active, primer extension was again performed but using a primer that hybridizes with the 5′-end of the *secG* mRNA (rnm014). A single fragment was extended from this primer as shown in Fig. 4b. The size of this fragment, as determined by comparison with the M13 sequence,

shows that its 5'-end matches the transcription start site (+1) of the *in silico* predicted promoter, clearly showing that this promoter is active and drives the expression of a transcript that includes *secG*.

Taken together these results indicate that the pneumococcal *rnr* transcript is expressed as part of an operon that includes *secG* and *smpB*. Processing of the operon to yield the mature gene products is likely to occur. Since we were not able to identify any other active promoter upstream of *rnr*, we believe that transcription of *rnr* and *smpB* does not occur independently and is most probably driven by the promoter identified upstream of *secG*.

SmpB mRNA and Protein Levels are Modulated by RNase R

We have just seen that in *S. pneumoniae rnr* is co-transcribed with *smpB*. In *E. coli* processing of tmRNA, the other main constituent of the trans-translation system is dependent on RNase R (Cairrao et al., 2003). The enzyme has also been involved in tmRNA degradation in *C. crescentus* and *P. syringae* (Hong et al., 2005; Purusharth et al., 2007). On the other hand, SmpB was shown to modulate the stability of *E. coli* RNase R (Liang and Deutscher, 2010). Thus, we were interested in clarifying which could be the involvement of RNase R with each of the main components of the trans-translation system in *S. pneumoniae*. For this purpose we compared both *smpB* and tmRNA expression between the wild-type and an isogenic mutant lacking RNase R (RNase R-) by Northern blot and/or RT-PCR. The results showed that the accumulation of the tmRNA precursor form (pre-tmRNA) at 15 °C is similar in both strains (Fig. 5a). Hence, RNase R from *S. pneumoniae* does not seem to be involved in the tmRNA processing under cold-shock contrary to that observed in *E. coli* (Cairrao et al., 2003). Nonetheless, in the absence of RNase R, a strong increase of *smpB* mRNA levels was observed (Fig. 5b). Interestingly this was mainly observed under cold-shock, which corresponds to the condition where RNase R is highly expressed. This data strongly indicates

that RNase R may be involved in the degradation of *smpB*. To check if the increment observed at the RNA levels would influence the final levels of protein in the cell, we analyzed the expression of SmpB under the same conditions. SmpB expression was compared by Western blot in the wild type and the RNase R-mutant derivative. In order to raise antibodies against pneumococcal SmpB, the protein was first cloned, overexpressed in fusion with tmRNA to avoid problems with solubility according to previous observations (Sundermeier et al., 2008), and then purified (see Material and Methods). Analysis of SmpB levels using these specific antibodies showed a significant increase in the protein levels in the absence of RNase R (Fig. 5b). However, contrary to the RNA levels, which were higher under cold-shock, we observed almost the same protein levels whether at 15 °C or 37 °C with even a slight increase at 37 °C. Together, these results strongly suggest that RNase R has a role in *smpB* degradation, which is determinant for the final levels of SmpB in the cell.

Discussion

RNase R levels are known to increase under certain stress situations (Andrade et al., 2006; Cairrao et al., 2003; Chen and Deutscher, 2005). This enzyme was shown to be important for growth and viability of some bacteria under cold shock (Cairrao et al., 2003; Charpentier et al., 2008; Erova et al., 2008; Purusharth et al., 2007; Reva et al., 2006), a condition where its levels are markedly augmented. In this report we have studied the regulation of the RNase R expression and the involvement of this exoribonuclease with the components of the trans-translation system in the human pathogen *S. pneumoniae*. Our results show that, as occurs in *E. coli*, pneumococcal RNase R is also induced after a downshift from 37 °C to 15 °C. According to our data, both mRNA and protein levels are highly elevated after cold-shock treatment, which could suggest that

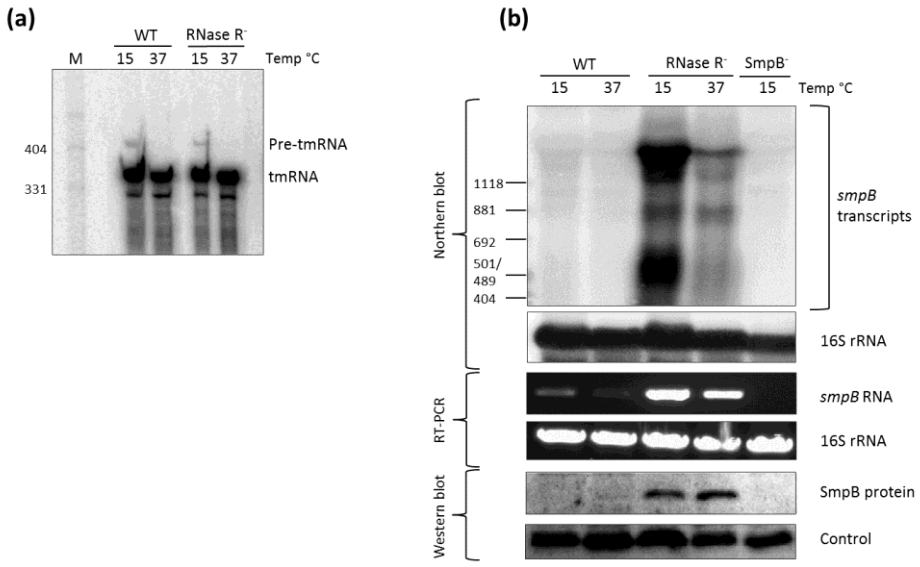

Fig. 4 - SmpB and tmRNA levels in the absence of RNase R. Northern blot, RT-PCR and Western blot analysis of RNA and protein samples extracted from wt and mutant strains as indicated on top of each lane. (a) Analysis of tmRNA by Northern blot. 15 μg of RNA extracted from the wild type (WT) and RNase R- mutant at 15 °C and 37 °C were separated on a 6 % polyacrylamide/8.3M urea. The gel was then blotted to a Hybond-N+ membrane and hybridized with a tmRNA specific riboprobe. (b) Analysis of SmpB protein (~18 kDa) and mRNA levels. (Upper panel) 15 μg of total RNA extracted in the same conditions were separated in an agarose 1.3 % gel, transferred to a Hybond-N+ membrane and hybridized with a specific probe for smpB. The membrane was stripped and then probed for 16S rRNA as loading control. (Middle panel) RT-PCR was performed on 50 ng of RNA from the same samples, using specific primers for *smpB*. The same experiments performed with primers specific for 16s rRNA show that there were no variations in the amount of total RNA used in the RT-PCR. (Lower panel) SmpB protein levels were analysed by Western immunoblotting with SmpB specific antibodies. 20 μg of total protein samples extracted in the same conditions were separated in a 10 % tricine-SDS polyacrylamide gel and blotted to nitrocellulose membrane. SmpB detection was carried using specific antibodies. A non-specific band (Control) detected with the same antibodies was used as loading control.

the higher levels of protein would be directly related with the increased amount of mRNA molecules in the cell. However, the expression of RNase R seems also to be modulated by SmpB. In the absence of this protein the levels of RNase R at 37 °C remain high and the temperature-controlled expression observed in the wild type seems to be lost in the SmpB- strain. This result resembles the *E. coli* situation recently reported (Liang and Deutscher, 2010) where RNase R was shown to be destabilized by SmpB during exponential phase in a tmRNA-dependent manner. Our data suggests that SmpB may also have an important role in the control of RNase R stability in *S. pneumoniae*. In *E. coli* the control of RNase R stability by SmpB was only reported in exponential phase. Subtle structural differences between two forms of RNase R (in exponential versus stationary phase) were hypothesized to account for the protein stability in stationary phase and under stressful conditions (Liang and Deutscher, 2010). It seems reasonable to speculate that in *S. pneumoniae*, the same structural differences that would stabilize the enzyme could occur under cold-shock. The control of RNase R stability by SmpB was shown to rely on a direct protein-protein interaction that involves the C-terminal region of RNase R and is enhanced by tmRNA (Liang and Deutscher, 2010). Interestingly, this unique lysine-rich domain of RNase R is essential both for recruitment of RNase R to ribosomes that are stalled on non-stop RNAs and for the activity of the enzyme on the selective degradation of these defective transcripts (Ge et al., 2010). A proper engagement of RNase R is dependent on both functional SmpB and tmRNA, and seems to be determinant for the enzyme's role in non-stop mRNA decay. We have analyzed pneumococcal RNase R sequence and also identified a lysine-rich C-terminal domain, which could mediate a direct association between RNase R and SmpB. All these observations point to a direct interaction between pneumococcal RNase R and SmpB, which may destabilize the exoribonuclease. However, we believe that the strong increment of the *rnr* mRNA levels detected at 15 °C may also account for the final

expression levels of RNase R in the cell. A higher amount of mRNA may compensate the low translation levels under cold-shock.

One of the first indications for the involvement of *E. coli* RNase R in the quality control of proteins was its association with a ribonucleoprotein complex involved in ribosome rescue (Karzai and Sauer, 2001). The enzyme was subsequently shown to be required for the maturation of *E. coli* tmRNA (Cairrao et al., 2003), one of the main components of the trans-translation system, and for its turnover in *C. crescentus* and *P. syringae* (Hong et al., 2005; Purusharth et al., 2007). Additional evidences included a direct role in the selective degradation of non-stop mRNAs (Ge et al., 2010; Richards et al., 2006) and destabilization of the enzyme by SmpB (Liang and Deutscher, 2010). In this work we strengthen the functional relationship between RNase R and the trans-translation machinery by demonstrating that RNase R is also implicated in the modulation of SmpB levels. A marked increase of both *smpB* mRNA and SmpB protein was observed in a strain lacking RNase R. The increment in mRNA levels is particularly high at 15 °C, the same condition where RNase R expression is higher. This fact suggests that the enzyme may be implicated in the control of *smpB* mRNA levels. The higher *smpB* mRNA levels detected at 15 °C could also suggest a temperature-dependent regulation of this message. However, the final levels of SmpB protein in the RNase R- strain were practically the same under cold-shock or at 37 °C. Translational arrest caused by the temperature downshift may be responsible for the difference between the protein and RNA levels. Alternatively, we may speculate that the interaction between RNase R and SmpB could mediate SmpB destabilization as well. This hypothesis would imply that RNase R/SmpB protein-protein association would direct both proteins for degradation. Further work is however, necessary to investigate this attractive possibility.

Analysis of the *S. pneumoniae* genome revealed the presence of two genes in the vicinity of the RNase R coding region, *secG* and *smpB*. Interestingly we

show that *rnr* and *smpB* are co-transcribed, being included in the same operon. Identification of several processing sites in the overlapping region between *rnr* and *smpB* indicates that this message is then processed, yielding either *rnr* or *smpB* single transcripts. We were not able to identify any active promoter immediately upstream of *rnr* or *smpB*. Several attempts to identify a promoter that could drive the transcription of the operon only allowed mapping a promoter upstream of *secG*, which is located immediately upstream of *rnr*. Indeed, we demonstrate that the *secG* promoter is active and most probably drives the expression of an operon that includes the three gene products: *secG*, *rnr* and *smpB*.

Comparison of the *rnr* genomic region of different Gram-negative and Gram-positive bacteria revealed that this genomic organization seems to be a common feature among Gram-positive bacteria (Table 2). The *rnr* gene is clustered with *secG* and *smpB* in numerous bacteria. Does this close localization have a biological meaning? It is known that bacterial genes involved in the same pathway are frequently co-localized (Overbeek et al., 2000). What could then be the physiological significance of the SecG association with two proteins involved in the trans-translation system? SecG is an integral membrane protein that is part of the SecYEG complex involved in the recognition and translocation of appropriate polypeptides through the membrane (see recent reviews (Driessen and Nouwen, 2008; du Plessis et al., 2011; Papanikou et al., 2007)). Recent data has suggested that trans-translation might be linked with other crucial co-translational processes, such as protein folding and secretion (Hayes and Keiler, 2010). Indeed, problems with nascent polypeptide folding were recently shown to target the translation complex to tmRNA (Ruhe and Hayes, 2010). This new hypothesis may provide a plausible explanation for the wide array of phenotypes associated with inactivation of tmRNA or SmpB (Keiler, 2007). Most bacterial

proteins are secreted through the SecYEG translocator, either during or after translation.

Table 2 – Organization of the RNase R genomic region in some Gram+ and Gram- bacteria. *secG*, *rnr* and *smpB* genes are highlighted.

Gram +	*Streptococcus pneumoniae*	***secG*-*rnr*-*smpB***
	Bacillus subtilis	***secG*-*yvaK*-*rnr*-*smpB*-*ssrA***
	Listeria monocytogenes	***secG*-*LMHCC_0148*-*rnr*-*smpB***
	Staphylococcus aureus	***secG*-*SAB0735*-*rnr*-*smpB***
	Clostridium botulinum	***secG*-*rnr*-*surE*-*smpB***
	Lactobacillus acidophilus	***secG*-*rnr*-*smpB***
	Enterococcus faecalis	***secG*-*EF2619*-*EF2618*-*rnr*-*smpB***
Gram -	*Escherichia coli*	*nsrR*-***rnr***-*rlmB*-*yjfI*[a]
	Salmonella typhimurium	*yjeT*-*purA*-*yjeB*-***rnr***-*yjfH*-*yjfI*
	Pseudomonas aeruginosa	***rnr***-*PA4936*-*rpsF*

[a] *nsrR* is the first gene of the operon according to Cairrão *et al.* [11].

When a translocator is blocked in a nascent polypeptide, SecY is degraded, which can be lethal or severely impair cell growth because this protein is required to assemble new translocators (van Stelten et al., 2009). An attractive model for a role of tmRNA in releasing blocked Sec translocators postulates that trans-translation activity over a ribosome stalled on a non-stop mRNA during co-translational translocation would allow a tagged protein to be translocated, saving SecY from destruction (Hayes and Keiler, 2010). The subcellular localization of tmRNA and SmpB is also consistent with a link between trans-translation and protein secretion. tmRNA and SmpB are concentrated in a helix-

like structure similar to that observed for SecY, SecE, and SecG (Campo et al., 2004; Russell and Keiler, 2009; Shiomi et al., 2006). The close genomic location of *secG*, *smpB* and *rnr* uncovered in this work also points to a functional relationship. This interesting possibility certainly deserves further investigation.

Materials and Methods

Bacterial Growth Conditions

E. coli was cultivated in Luria-Bertani broth (LB) at 37 °C with agitation, unless differently specified. When required, growth medium was supplemented with 100 μg/ml ampicillin (Amp). *S. pneumoniae* strains were grown in Todd Hewitt medium, supplemented with 0.5 % yeast extract (THY) at 37 °C without shaking, except when differently described. Growth medium was supplemented with 3 μg/ml chloramphenicol (Cm) or 250 μg/ml kanamycin (Kan) when required.

Oligonucleotides, Bacterial Strains and Plasmids

All oligonucleotides used in this work are listed in Table S1 and were synthesized by STAB Vida, Portugal. *E. coli* strains used in this work are listed in Table 1. All *S. pneumoniae* strains are isogenic derivatives of the JNR7/87 capsulated strain – TIGR4 (Tettelin et al., 2001) and are also listed in Table 1.

The *S. pneumoniae* smpB- deficient mutant was created through allelic replacement mutagenesis (Song et al., 2005) using a DNA fragment containing the *smpB* flanking regions, in which *smpB* is replaced by a kanamycin resistance cassette. *kan* marker was amplified from pR410 (Sung et al., 2001) with primers smd019 and smd020. The upstream and downstream *smpB* flanking regions were amplified by PCR using respectively the primer pairs smd053/smd054 and smd055/smd056. smd054 and smd055 contained 3′ extensions complementary

Table 1 – List of strains used in this work.

Strain	Relevant markers/Genotype	Source/Reference
E. coli DH5α	**F' fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17a**	[52]
E. coli DH5α pSDA-02	***E. coli* DH5α carrying pSDA-02**	**This work**
E. coli BL21(DE3)	**F– *omp*T *gal dcm lon hsd*S_B(r_B- m_B-) λ(DE3 [*lac*I *lac*UV5-T7 gene 1 *ind*1 *sam*7 *nin*5])**	[53]
E. coli BL21(DE3)his*rnr*	***E. coli* BL21(DE3) overexpressing pneumococcal His-tagged RNase R**	[30]
E. coli BL21(DE3) pSDA-02	***E. coli* BL21(DE3) carrying pSDA-02**	**This work**
S. pneumoniae JNR7/87 (TIGR4)		[45]
S. pneumoniae TIGR4 RNase R-	**TIGR4 *rnr*- (Δ*rnr*-Cm^R)**	**C. Arraiano and P. Lopez Labs[a]**
S. pneumoniae TIGR4 SmpB-	**TIGR4 *smpB*- (Δ*smpB*-Kan^R)**	**This work**

[a] A chloramphenicol-resistance cassette replaces nucleotides +1 to +2288 of the *rnr* gene (Mohedano, Domingues *et al*., manuscript in preparation)

to the 5′- and 3′- ends of the *kan* marker, respectively. The combination of these three PCR products was used as template in other PCR reaction performed with the primers smd053 and smd056. The resulting PCR product corresponded to a ~3.9 kb fragment containing the *smpB* flanking genes (~1.5 kb each side) and a *kan* marker replacing nucleotides +38 to +467 of the *smpB* gene. This fragment was used to transform TIGR4 competent cells of *S. pneumoniae*. Competent cultures of *S. pneumoniae* TIGR4 were prepared in Todd- Hewitt medium (TH) plus 0.5 % glycine and 0.5 % yeast extract by several cycles of dilutions and growing at 37 °C up to an OD at 650 nm of 0.3. Competent cells were then grown in a casein hydrolase-based medium (AGCH) with 0.2 % sucrose (Suc) and 0.001 % CaCl2

containing 1.5 x 107 CFU/ml and treated with 100 ng/ml of CSP-2 for 14 min at 30 °C. Then 590 ng of DNA were added, and the culture was incubated at 30 °C for 40 min. The culture was then transferred to 37 °C and incubated for 120 min before plating on media plates (AGCH medium with 1 % agar plus 0.3 % Suc and 0.2 % yeast extract) containing 250 μg/ml Kan. Transformants were grown at 37 °C in a 5 % CO2 atmosphere. A Kan^R transformant was selected, and the insertion/deletion mutation was confirmed by DNA sequencing at the Genomic Service of Instituto de Salud Carlos III.

E. coli SmpB overexpressed in the absence of tmRNA is insoluble (Sundermeier et al., 2008). Hence, in order to overexpress and purify pneumococcal SmpB, its coding region was cloned in fusion with pneumococcal *ssrA* (the gene encoding tmRNA) to allow co-expression of both. *smpB* was amplified by PCR with primers rnm010 and rnm011, and contains a 3′ extension complementary to the 5′-end of *ssrA*. *ssrA* was amplified using the primer pair smd057/smd058. The two PCR fragments were then mixed and used as template in a PCR with primers rnm010 and smd058. All amplification reactions were carried out with Phusion DNA polymerase (Finzzymes). The resulting PCR product was digested with NdeI and BamHI (Fermentas), and cloned into the pET-15b vector (Novagen) previously cleaved with the same restriction enzymes. This construction, named pSDA-02, was first obtained in *E. coli* DH5α and then transferred to *E. coli* BL21(DE3) to allow the expression of His-SmpB. This construct was confirmed by DNA sequencing at STAB Vida, Portugal.

Overexpression and Purification of Proteins

RNase R from *S. pneumoniae* was purified as previously described (Domingues et al., 2009). For purification of SmpB, BL21(DE3) cells containing pSDA-02 plasmid were grown at 37 °C in 250 ml of LB medium supplemented with 100 μg/ml Amp to an OD600 of 0.5. Overexpression of SmpB was then

induced by addition of 1 mM IPTG; induction proceeded for 3 hours at 37 °C. Cells were harvested by centrifugation and stored at -80 °C. Purification was performed by histidine affinity chromatography using HisTrap Chelating HP columns (GE Healthcare) and AKTA HPLC system (GE Healthcare) as follows. Frozen cells were thawed and resuspended in lysis buffer (50 mM HEPES pH 7.5, 1 M NH4Cl, 5 mM MgCl2, 2 mM β-mercaptoethanol, 10 mM imidazole). Cell suspensions were lysed using a French Press at 9000 psi in the presence of 1 mM PMSF. The crude extracts were treated with Benzonase (Sigma) to degrade the nucleic acids and clarified by a 30 min centrifugation at 10000 xg. The clarified extracts were then loaded onto a HisTrap Chelating Sepharose 1 ml column equilibrated with buffer A (20 mM sodium phosphate pH 7.4, 0,5 M NaCl, 20 mM imidazole). Protein elution was achieved by a continuous imidazole gradient (from 20 mM to 500 mM) in buffer A. The fractions containing the purified protein were pooled together and concentrated by centrifugation at 4 °C in an Amicon Ultra Centrifugal Filter Device with a molecular mass cutoff of 10 kDa (Millipore). Protein concentration was determined using Bradford method (Bradford, 1976). SmpB and RNase R purified proteins were loaded in a SDS-PAGE gel and Coomassie blue stained for band excision (data not shown). Bands corresponding to a total of 500 μg of each protein were used to raise antibodies against the respective pneumococcal proteins (Eurogentec).

RNA Extraction and Northern Blotting

Overnight cultures of *S. pneumoniae* TIGR4 wild type and mutant derivatives were diluted in pre-warmed THY to a final OD600 of 0.1, and incubated at 37 °C until OD600 ~ 0.3. At this point, cultures were split in two aliquots and each aliquot was further incubated at 15 °C or 37 °C for 2 h. 20 ml culture samples were collected, mixed with 1 volume of stop solution (10 mM Tris pH 7.2, 25 mM NaNO3, 5 mM MgCl2, 500 μg/ml chloramphenicol) and

harvested by centrifugation (10 min, 2800 xg, 4 ºC). Total RNA was extracted using Trizol reagent (Ambion) essentially as described by the manufacturer, with some modifications. Pneumococcal cells were lysed by incubation in 650 µl lysis buffer (sodium citrate 150 mM, saccharose 25 %, sodium deoxicolate 0.1 %, SDS 0.01 %) for 15 min at 37 °C followed by addition of 0.1 % SDS. After lysis, samples were treated with 10 U Turbo DNase (Ambion) for 1 h at 37 °C. After extraction, the RNA integrity was evaluated by gel electrophoresis and its concentration determined using a Nanodrop 1000 machine (Nanodrop Technologies).

For Northern blot analysis, total RNA samples were separated under denaturating conditions either by a 6 % polyacrylamide / urea 8.3 M gel in TBE buffer or by 1.3 % agarose MOPS/formaldehyde gel. For polyacrylamide gels, transfer of RNA onto Hybond-N+ membranes (GE Healthcare) was performed by electroblotting (1 h 50 min, 24 V, 4 °C) in TAE buffer. For agarose gels RNA was transferred to Hybond-N+ membranes by capillarity using 20×SSC as transfer buffer. In both cases, RNA was UV cross-linked to the membrane immediately after transfer. Membranes were then hybridized in RapidHyb Buffer (GE Healthcare) for 16 h at 68 °C for riboprobes and 43 °C in the case of oligoprobes. After hybridization, membranes were washed as described (Viegas et al., 2007). Signals were visualized by PhosphorImaging (Storm Gel and Blot Imaging System, Amersham Bioscience) and analyzed using the ImageQuant software (Molecular Dynamics).

Hybridization Probes

Riboprobe synthesis and oligoprobe labeling was performed as previously described (Viegas et al., 2007). PCR products used as template in the riboprobe synthesis were obtained using the following primer pairs: rnm007/seqT4-3 for *rnr*, T7tmRNA/P2tmRNA for tmRNA and smd041T7/smd040

for *smpB*. The DNA probe for 16S rRNA was generated using the primer 16sR labeled at 5′ end with [γ-32P]ATP using T4 polynucleotide kinase (Fermentas).

Reverse Transcription-PCR (RT-PCR)

RT-PCR reactions were carried out using total RNA, with the OneStep RT-PCR kit (Qiagen), according to the supplier's instructions. The primer pairs seqT4-2/seqT4-3 and rnm010/smd041 were used to analyze *rnr* and *smpB* expression, respectively. Amplification of *secG+rnr* and *rnr+smpB* fragments was performed with the primer pairs smd038/smd050 and smd064/smd041, respectively. The position of these primers in *S. pneumoniae* genome is indicated in Figure 1. As an independent control, 16S rRNA was amplified with specific primers 16sF/16sR. Prior to RT-PCR, all RNA samples were treated with Turbo DNA free Kit (Ambion). Control experiments, run in the absence of reverse transcriptase, yielded no product.

Primer Extension Analysis

Total RNA was extracted as described above. Primers rnm016, rnm014 and rnm002, respectively complementary to the 5′-end of *rnr*, *secG* and *smpB*, were 5′-end-labeled with [γ-32P]ATP using T4 polynucleotide kinase (Fermentas). Unincorporated nucleotides were removed using a MicroSpinTM G-25 Column (GE Healthcare). 2 pmol of labeled primer was annealed to 5 μg of RNA, and cDNA was synthesized using 10U of Transcriptor Reverse Transcriptase (Roche). M13 sequencing reaction was performed with Sequenase Version 2.0 sequencing kit (USB) according to the supplier instructions. The primer extension products were separated in parallel with the M13 sequencing reaction on a 5 % polyacrylamide / urea 8 M sequencing gel. The gel was exposed and signals were visualized in a PhosphorImager (Storm Gel and Blot Imaging System, Amersham Bioscience).

Total Protein Extraction and Western Blotting

Cell cultures used to prepare protein extracts were grown in the same conditions as described above for RNA extraction. 20 ml culture samples were collected, mixed with 1 volume of stop solution [10 mM Tris (pH 7.2), 25 mM NaNO3, 5 mM MgCl2, 500 μg/ml chloramphenicol] and harvested by centrifugation (10 min, 2800 xg, 4 °C). The cell pellet was resuspended in 100 μl TE buffer supplemented with 1 mM PMSF, 0.15 % sodium deoxicolate and 0.01 % SDS. After 15 min incubation at 37 °C, SDS was added to a final concentration of 1 %. Protein concentration was determined using a Nanodrop 1000 machine (NanoDrop Technologies). 20 μg of total protein were separated in a 7 % (for RNase R detection) or 10 % (for SmpB detection) tricine-SDS-PAGE gel, following the modifications described by (Haider et al., 2010). After electrophoresis, proteins were transferred to a nitrocellulose membrane (Hybond ECL, GE Healthcare) by electroblotting using the Trans-Blot SD semidry electrophoretic system (Bio-Rad). Membranes were then probed with a 1:1000 or 1:500 dilution of anti-SmpB or anti-RNase R antibodies, respectively. ECL anti-rabbit IgG peroxidase conjugated (Sigma) was used as the secondary antibody in a 1:10000 dilution. Immunodetection was conducted via a chemiluminescence reaction using Western Lightning Plus-ECL Reagents (PerkinElmer).

Promoter Prediction

In silico predictions of putative promoters were performed using the BPROM SoftBerry software (http://linux1.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb) and Neural Network Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.html) (Reese, 2001) bioinformatics tools.

Acknowledgments

We thank Andreia Aires for technical assistance. Fundação para a Ciência e Tecnologia gave financial support to R. Moreira (Doctoral fellowship), S. Domingues (Postdoctoral fellowship) and S. Viegas (Postdoctoral fellowship). This work was supported by Fundação para a Ciência e Tecnologia (FCT) in Portugal and by the Fondo de Investigación Sanitaria (FIS) (PI08/0442), CIBER Enfermedades Respiratorias (initiative of the Instituto de Salud Carlos III) in Spain, and by the Bilateral Collaboration program between Conselho Reitores Universidades Portuguesas (CRUP) from Portugal and Ministerio de Ciencia e Innovación (MICINN) (HP2008-0041) Acciones Integradas of Spain.

References

Andrade, J.M., F. Cairrao, and C.M. Arraiano. 2006. RNase R affects gene expression in stationary phase: regulation of ompA. *Mol Microbiol*. 60:219-228.

Andrade, J.M., V. Pobre, I.J. Silva, S. Domingues, and C.M. Arraiano. 2009. The role of 3'-5' exoribonucleases in RNA degradation. *Prog Mol Biol Transl Sci*. 85:187-229.

Arraiano, C.M., J.M. Andrade, S. Domingues, I.B. Guinote, M. Malecki, R.G. Matos, R.N. Moreira, V. Pobre, F.P. Reis, M. Saramago, I.J. Silva, and S.C. Viegas. 2010. The critical role of RNA processing and degradation in the control of gene expression. *FEMS Microbiol Rev*. 34:883-923.

Arraiano, C.M., and L.E. Maquat. 2003. Post-transcriptional control of gene expression: effectors of mRNA decay. *Mol Microbiol*. 49:267-276.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 72:248-254.

Cairrao, F., A. Cruz, H. Mori, and C.M. Arraiano. 2003. Cold shock induction of RNase R and its role in the maturation of the quality control mediator SsrA/tmRNA. *Mol Microbiol*. 50:1349-1360.

Campo, N., H. Tjalsma, G. Buist, D. Stepniak, M. Meijer, M. Veenhuis, M. Westermann, J.P. Muller, S. Bron, J. Kok, O.P. Kuipers, and J.D. Jongbloed. 2004. Subcellular sites for bacterial protein export. *Mol Microbiol*. 53:1583-1599.

Campos-Guillen, J., J.L. Arvizu-Gomez, G.H. Jones, and G. Olmedo-Alvarez. 2010. Characterization of tRNA(Cys) processing in a conditional Bacillus subtilis CCase mutant reveals the participation of RNase R in its quality control. *Microbiology*. 156:2102-2111.

Charpentier, X., S.P. Faucher, S. Kalachikov, and H.A. Shuman. 2008. Loss of RNase R induces competence development in Legionella pneumophila. *J Bacteriol*. 190:8126-8136.

Chen, C., and M.P. Deutscher. 2005. Elevation of RNase R in response to multiple stress conditions. *J Biol Chem*. 280:34393-34396.

Cheng, Z.F., and M.P. Deutscher. 2002. Purification and characterization of the Escherichia coli exoribonuclease RNase R. Comparison with RNase II. *J Biol Chem*. 277:21624-21629.

Cheng, Z.F., and M.P. Deutscher. 2003. Quality control of ribosomal RNA mediated by polynucleotide phosphorylase and RNase R. *Proc Natl Acad Sci U S A*. 100:6388-6393.

Cheng, Z.F., and M.P. Deutscher. 2005. An important role for RNase R in mRNA decay. *Mol Cell*. 17:313-318.

Cheng, Z.F., Y. Zuo, Z. Li, K.E. Rudd, and M.P. Deutscher. 1998. The vacB gene required for virulence in Shigella flexneri and Escherichia coli encodes the exoribonuclease RNase R. *J Biol Chem*. 273:14077-14080.

Condon, C., and H. Putzer. 2002. The phylogenetic distribution of bacterial ribonucleases. *Nucleic Acids Res*. 30:5339-5346.

Domingues, S., R.G. Matos, F.P. Reis, A.M. Fialho, A. Barbas, and C.M. Arraiano. 2009. Biochemical characterization of the RNase II family of exoribonucleases from the human pathogens Salmonella typhimurium and Streptococcus pneumoniae. *Biochemistry*. 48:11848-11857.

Driessen, A.J., and N. Nouwen. 2008. Protein translocation across the bacterial cytoplasmic membrane. *Annu Rev Biochem*. 77:643-667.

du Plessis, D.J., N. Nouwen, and A.J. Driessen. 2011. The Sec translocase. *Biochim Biophys Acta*. 1808:851-865.

Erova, T.E., V.G. Kosykh, A.A. Fadl, J. Sha, A.J. Horneman, and A.K. Chopra. 2008. Cold shock exoribonuclease R (VacB) is involved in Aeromonas hydrophila pathogenesis. *J Bacteriol*. 190:3467-3474.

Fonseca, P., R. Moreno, and F. Rojo. 2008. Genomic analysis of the role of RNase R in the turnover of Pseudomonas putida mRNAs. *J Bacteriol*. 190:6258-6263.

Ge, Z., P. Mehta, J. Richards, and A.W. Karzai. 2010. Non-stop mRNA decay initiates at the ribosome. *Mol Microbiol*. 78:1159-1170.

Grossman, D., and A. van Hoof. 2006. RNase II structure completes group portrait of 3' exoribonucleases. *Nat Struct Mol Biol*. 13:760-761.

Haider, S.R., H.J. Reid, and B.L. Sharp. 2010. Modification of tricine-SDS-PAGE for online and offline analysis of phosphoproteins by ICP-MS. *Anal Bioanal Chem*. 397:655-664.

Hayes, C.S., and K.C. Keiler. 2010. Beyond ribosome rescue: tmRNA and co-translational processes. *FEBS Lett*. 584:413-419.

Hong, S.J., Q.A. Tran, and K.C. Keiler. 2005. Cell cycle-regulated degradation of tmRNA is controlled by RNase R and SmpB. *Mol Microbiol*. 57:565-575.

Karzai, A.W., and R.T. Sauer. 2001. Protein factors associated with the SsrA.SmpB tagging and ribosome rescue complex. *Proc Natl Acad Sci U S A*. 98:3040-3044.

Keiler, K.C. 2007. Physiology of tmRNA: what gets tagged and why? *Curr Opin Microbiol*. 10:169-175.

Keiler, K.C. 2008. Biology of trans-translation. *Annu Rev Microbiol*. 62:133-151.

Lalonde, M.S., Y. Zuo, J. Zhang, X. Gong, S. Wu, A. Malhotra, and Z. Li. 2007. Exoribonuclease R in Mycoplasma genitalium can carry out both RNA processing and degradative functions and is sensitive to RNA ribose methylation. *RNA*. 13:1957-1968.

Liang, W., and M.P. Deutscher. 2010. A novel mechanism for ribonuclease regulation: transfer-messenger RNA (tmRNA) and its associated protein SmpB regulate the stability of RNase R. *J Biol Chem*. 285:29054-29058.

Oussenko, I.A., T. Abe, H. Ujiie, A. Muto, and D.H. Bechhofer. 2005. Participation of 3'-to-5' exoribonucleases in the turnover of Bacillus subtilis mRNA. *J Bacteriol*. 187:2758-2767.

Overbeek, R., N. Larsen, G.D. Pusch, M. D'Souza, E. Selkov, Jr., N. Kyrpides, M. Fonstein, N. Maltsev, and E. Selkov. 2000. WIT: integrated system for high-throughput genome sequence analysis and metabolic reconstruction. *Nucleic Acids Res*. 28:123-125.

Papanikou, E., S. Karamanou, and A. Economou. 2007. Bacterial protein secretion through the translocase nanomachine. *Nat Rev Microbiol*. 5:839-851.

Purusharth, R.I., B. Madhuri, and M.K. Ray. 2007. Exoribonuclease R in Pseudomonas syringae is essential for growth at low temperature and plays a novel role in the 3' end processing of 16 and 5 S ribosomal RNA. *J Biol Chem*. 282:16267-16277.

Reese, M.G. 2001. Application of a time-delay neural network to promoter annotation in the *Drosophila melanogaster* genome. *Comput Chem*. 26:51-56.

Reva, O.N., C. Weinel, M. Weinel, K. Bohm, D. Stjepandic, J.D. Hoheisel, and B. Tummler. 2006. Functional genomics of stress response in *Pseudomonas putida* KT2440. *J Bacteriol*. 188:4079-4092.

Richards, J., P. Mehta, and A.W. Karzai. 2006. RNase R degrades non-stop mRNAs selectively in an SmpB-tmRNA-dependent manner. *Mol Microbiol*. 62:1700-1712.

Richards, J., T. Sundermeier, A. Svetlanov, and A.W. Karzai. 2008. Quality control of bacterial mRNA decoding and decay. *Biochim Biophys Acta*. 1779:574-582.

Ruhe, Z.C., and C.S. Hayes. 2010. The N-terminus of GalE induces tmRNA activity in Escherichia coli. *PLoS One*. 5:e15207.

Russell, J.H., and K.C. Keiler. 2009. Subcellular localization of a bacterial regulatory RNA. *Proc Natl Acad Sci U S A*. 106:16405-16409.

Shiomi, D., M. Yoshimoto, M. Homma, and I. Kawagishi. 2006. Helical distribution of the bacterial chemoreceptor via colocalization with the Sec protein translocation machinery. *Mol Microbiol*. 60:894-906.

Song, J.H., K.S. Ko, J.Y. Lee, J.Y. Baek, W.S. Oh, H.S. Yoon, J.Y. Jeong, and J. Chun. 2005. Identification of essential genes in *Streptococcus pneumoniae* by allelic replacement mutagenesis. *Mol Cells*. 19:365-374.

Sundermeier, T., Z. Ge, J. Richards, D. Dulebohn, and A.W. Karzai. 2008. Studying tmRNA-mediated surveillance and nonstop mRNA decay. *Methods Enzymol*. 447:329-358.

Sung, C.K., H. Li, J.P. Claverys, and D.A. Morrison. 2001. An *rpsL* cassette, janus, for gene replacement through negative selection in *Streptococcus pneumoniae*. *Appl Environ Microbiol*. 67:5190-5196.

Tettelin, H., K.E. Nelson, I.T. Paulsen, J.A. Eisen, T.D. Read, S. Peterson, J. Heidelberg, R.T. DeBoy, D.H. Haft, R.J. Dodson, A.S. Durkin, M. Gwinn, J.F. Kolonay, W.C. Nelson, J.D. Peterson, L.A. Umayam, O. White, S.L. Salzberg, M.R. Lewis, D. Radune, E. Holtzapple, H. Khouri, A.M. Wolf, T.R. Utterback, C.L. Hansen, L.A. McDonald, T.V. Feldblyum, S. Angiuoli, T. Dickinson, E.K. Hickey, I.E. Holt, B.J. Loftus, F. Yang, H.O. Smith, J.C. Venter, B.A. Dougherty, D.A. Morrison, S.K. Hollingshead, and C.M. Fraser. 2001. Complete genome sequence of a virulent isolate of Streptococcus pneumoniae. *Science*. 293:498-506.

Tobe, T., C. Sasakawa, N. Okada, Y. Honma, and M. Yoshikawa. 1992. vacB, a novel chromosomal gene required for expression of virulence genes on the large plasmid of Shigella flexneri. *J Bacteriol*. 174:6359-6367.

Tsao, M.Y., T.L. Lin, P.F. Hsieh, and J.T. Wang. 2009. The 3'-to-5' exoribonuclease (encoded by HP1248) of Helicobacter pylori regulates motility and apoptosis-inducing genes. *J Bacteriol*. 191:2691-2702.

van Stelten, J., F. Silva, D. Belin, and T.J. Silhavy. 2009. Effects of antibiotics and a proto-oncogene homolog on destruction of protein translocator SecY. *Science*. 325:753-756.

Viegas, S.C., V. Pfeiffer, A. Sittka, I.J. Silva, J. Vogel, and C.M. Arraiano. 2007. Characterization of the role of ribonucleases in Salmonella small RNA decay. *Nucleic Acids Res*. 35:7651-7664.

Zuo, Y., and M.P. Deutscher. 2001. Exoribonuclease superfamilies: structural analysis and phylogenetic distribution. *Nucleic Acids Res*. 29:1017-1026.

Supplementary Data

Table S1 – List of oligonucleotides used in this work.

Oligo name	Sequence 5' to 3'
16sF	AGAGTTTGATCCTGGCTCAG
16sR	ACGGCTACCTTGTTACGACTT
P2tmRNA	GTCGTTACGGATTCGACAG
rnm002	TCACTTGAGCAAAGCCATCC
rnm007	GTTTTTTTTAATACGACTCACTATAGGGACATCGCTATAGGTCATACG
rnm010	GGAATTCCATATGGCAAAGGGCGAGGGAAAGGTC
rnm011	CTAATCTAAAGGCCACTTCCTTATCGCTGATTAACAGCTTTC
rnm014	GAACTGGCATCAAATACATTGCTGGATTGG
rnm016	CCCAAAGCCTGAGCCAAATCATTAACAGTC
seqt4-2	GACATCGCTATAGGTCATACG
seqt4-3	GTTTGACAACAGTTGTCGGG
smd019	GGGCCCGTTTGATTTTTAATG
smd020	GGTACTAAAACAATTCATCC
smd038	TTGAACGCAGTAAAGCTCGC
smd040	ATCGTAGATACGCTAGAGGCAGG
smd041	CATCACACGCGCGATATCTC
smd041T7	GTTTTTTTTAATACGACTCACTATAGGCATCACACGCGCGATATCTC
smd050	GCTTCTGCTGCTGTTCCCTTATTG
smd053	CGGGGTACCTGGGGTTCACATCGCAGATG
smd054	CATTAAAAATCAAACGGGCCCTTATTTTGTGCGACGACC
smd055	GGATGAATTGTTTTAGTACCAAAGAGGAATTGAAAATGGAAAAATTAG
smd056	CGCGGATCCCCGCGGAGATCCTGGTAAATC
smd057	GGAAGTGGCCTTTAGATTAG
smd058	CGCGGATCCTGGAGCCGGTGGGAGTCGAAC
smd064	CAGTCTAGTCGTAGTGGCAG
T7tmRNA	GTTTTTTTTTTAATACGACTCACTATAGGGAGGTGTCTACAACCATAGGTTATG

Chapter 6

Discussion and Future Perspectives

The regulation of bacterial transcription has been a topic of interest for several decades. Regulation of protein expression in bacteria largely occurs at the level of transcription. This regulation is often due to proteins that bind specific regions on the chromosome (promoters). It is known that transcription initiation is regulated by a number of DNA-binding proteins These proteins either bind specific sequences on DNA (activators or repressors) (for reviews, (Browning and Busby, 2004)), bind in a nonspecific manner (nucleoid-associated proteins) (for reviews, see (McLeod and Johnson, 2001)) or regulate by mechanisms that do not involve the direct interaction of transcription factors with DNA (reviewed by (Haugen et al., 2008)). The role of nucleoid proteins in controlling gene expression has become increasingly recognized over the past few years. They can modulate transcription in response to environmental signals by a variety of mechanisms. Their ability to alter DNA structure or directly interact with RNA polymerase is essential for their activity. The H-NS protein was described as a transcription regulator which affects σ^S-dependent genes (Barth et al., 1995). This protein is abundant in bacterial cells and is often compared to eukaryotic histones because of its high affinity for curved DNA rich in AT-rich regions (Dorman, 2004). Often, H-NS acts as a selective silencer of genes that rapidly respond to environmental changes (Barth et al., 1995; Lang et al., 2007; White-Ziegler and Davis, 2009). *bolA* gene is known to be promptly induced upon stresses, including temperature, osmotic shock and carbon starvation stresses (Santos et al., 1999). In this context we hypothesised a regulatory role of H-NS on *bolA* expression. We indeed observed that H-NS downregulates *bolA* expression. Moreover, we showed that H-NS regulation over *bolA* is direct and that this protein is acting in both *bolA* promoters. Interestingly, differences on the binding affinity were noticed when distinct fragments containing distinct *bolA* regulatory regions were used. Even though H-NS was able to bind bolAp1 and bolAp2, a partial loss of binding affinity was noticed when bolAp2 promoter was absent or when the ORF region

was present in the tested substrate. Since H-NS is sensitive to the DNA conformation (Dorman, 2004), the selective binding is probably due to the 3D conformation of the DNA. *bolA* promoter region presents a 3D conformation that resembles different examples of H-NS preferred targets. That is, a curved conformation (Dorman, 2004). However, this curved conformation is just noticed when both *bolA* promoters are present. Thus, we believe the reason for the lower affinity of the substrate lacking bolAp2 promoter is caused by the loss of the bending observed with both promoters. It was previously shown that *bolA* co-immunoprecitate with H-NS (Dorman, 2004). The reported observation is probably due to the direct interaction with *bolA* regulatory region. We showed that H-NS binds simultaneously to several sites within the entire promoter region of *bolA*, and forms higher-order structures originating a repressive nucleoprotein complex that modulates the activity of bolAp1 and bolAp2. Overall we revealed that the pleiotropic histone-like protein H-NS is a new transcription regulator of *bolA*. We confirmed that H-NS is directly repressing *bolA* expression by binding to different locations along its entire promoter regions probably changing the DNA conformation impeding the DNA polymerase binding. Moreover, the binding sites are confined to a curved DNA region, known to be the H-NS preferred consensus structure.

BolA has been shown to be a protein that affects several cellular functions. It has been described as a morphogene (Aldea et al., 1988; Freire et al., 2009; Guinote, 2011) important for cell survival (Freire et al., 2006). In this context, a fine tuned regulation of this gene may be essential for the cell. Since H-NS is known to be involved in the flagella biosynthesis (Bertin et al., 1994) influencing biofilms architecture (Wood et al., 2006), and *bolA* previously shown to be able to induce biofilm formation (Vieira et al., 2004), we may be close to find the link between motility and biofilm development. A role of BolA on the complex pathway of flagella and/or curli biosynthesis may be of reasonable enough

interest to give a step on the study of the possible relationship of the H-NS and the expression of BolA. Since BolA has an enormous impact in cell division and cell morphology, it is expected that the regulation of this gene implicates many other transcription factors and regulators in order to tightly regulate its transcription. Thus, the discovery of additional regulators of BolA is of a major concern.

As it was described above, BolA has been shown to be a pleiotropic protein that affects several cellular functions. Homologues of this protein are widespread in nature, including in eukaryotes, but curiously, it is absent in Gram-positive bacteria. Moreover, some organisms have more than one copy of this gene, for example, in *E. coli,* a homologue was recently described, the YrbA protein. YrbA has 23% of 66 aminoacid overall identity, and 58% of similarity at the BolA domain and over 70% of the aminoacid residues of both proteins perfectly align. Similarly, to BolA, YrbA has a helix-turn-helix motif, usually responsible for DNA-protein interaction. Until very recently, BolA was never shown to be a DNA-binding protein even though NMR structure of a BolA-like protein in Mus musculus allowed the identification of a characteristic helix-turn-helix (HTH) motif suggesting a possible DNA-protein interaction function for this protein (Kasai et al., 2004). Clarifying the role of BolA in the cell is of major interest. The main phenotype observed when is overexpressed is the ability to shorten the cells from bacilli to spheres. The mechanism behind this observation was not described neither BolA was directly associated with any cell division crucial element. In order to attempt to connect *bolA* expression with cell division, different approaches were taken, including using different antibiotics to block important penicillin binding proteins (PBPs) known to be involved in the cell division phenomena. When cell septum formation was arrested, we observed that BolA was not able to shorten the cells like in a normal growth situation, but it arrested further filamentation. However, if the BolA induction happened before blocking

septum formation, the expected filamentation was not observed. The absence of *bolA*-mediated morphology when septation is inhibited before *bolA* induction raised two different hypothesis: a possible irreversible morphological pathway and BolA is no longer able to induce a rounder shape or BolA-dependent inhibition of elongation might require functional septation machinery. Cefmetazole was used to inhibit all *E. coli* PBPs except PBP2, the unique penicillin binding protein that is not inhibited by this antibiotic (Ohya et al., 1978). This allowed us to focus on the effect of *bolA* on elongation mechanisms, independently of PBP5 or PBP6, previously shown to be regulated by BolA (Santos et al., 2002). In fact, it was verified that *bolA* could affect morphology independently of PBP5 and/or PBP6. Therefore, BolA overexpression either blocked PBP2-dependent cell elongation or affected another mechanism involved in the normal rod shape maintenance and essential for elongation. Overexpression of PBP2 was shown to be unable to revert the round phenotype caused by *bolA* overexpression (Aldea et al., 1988). Besides the important role of PBP2 in bacterial cell elongation, MreB, a structural homologue of actin, is essential for cell elongation and maintenance of the rod shape (Jones et al., 2001; van den Ent et al., 2001). MreB polymerises to form a spiral structure along the interior of the cell wall (Jones et al., 2001). Interestingly, similarly to *bolA* overexpression phenotype, mutations of the *mreB* gene or specific inhibition of MreB cause round morphology and spherical cells (van den Ent et al., 2001). One of the most interesting outcomes of this study was, obtained when analysed the effect of *bolA* in MreB spatial organization. When the expression levels of BolA increased, no more MreB filaments could be detected. Therefore, BolA overexpression affected MreB filaments. The process of MreB filament formation is probably dependent on the intracellular levels of MreB, in a way similar to what happens in the actin polymerization process (Korn et al., 1987). If *bolA* considerably lowers the expression levels of MreB, this could be interfering with

the architecture of MreB polymers. In fact, when BolA was present in high amounts in the cell, MreB protein and mRNA levels were significantly lower, showing that this *E. coli* morphogene acted as a new negative regulator of MreB. Moreover, this regulation was verified to be direct over *mreBCD* promoters. The finding that BolA can directly repress the transcription of *mreBCD* and lower the levels of MreB in the cell presents a broad impact on cellular features, such as morphology maintenance and elongation mechanisms, especially in stress conditions when *bolA* is induced. It is also a major step toward understanding the regulation of MreB expression, a protein responsible for the cytoskeleton, an essential architectural element of the bacterial cell. However, MreB is not the unique protein essential for the typical rod shape of *E. coli.* For instance, the recently described protein RodZ, showed to be as important as MreB in this process. RodZ is involved in the maintenance of cell shape through interaction with the MreB cytoskeleton (Alyahya et al., 2009; Bendezu et al., 2009; Shiomi et al., 2008). Like MreB, it is distributed along the cell in a helical pattern and is required for the proper formation of MreB spirals. What will be the effect of BolA on RodZ? Still, as a distinct subject of interest is the intriguing fact of BolA being absent in Gram-positive bacteria. Is that related with the different mechanisms of cell division? To evaluate this, we plan to clone *bolA* in a model Gram-positive bacterium, such as *B. subtilis,* and evaluate the effects of the gene expression in the bacteria.

The influence of BolA in different proteins involved in the cell division apparatus or cell membrane homeostasis is still enigmatic and needs further studies. In order to better understand the global role of BolA as a transcription factor, we performed microarrays. In LB media and exponential phase, the effects of BolA are not noticeable like they are in stationary phase. Nevertheless, in case of the cells that enter in a stress condition, like osmotic shock, temperature alteration or nutrients depravation, BolA is induced and responds to the stress

like it does in stationary phase. To simulate a stress response in exponential phase, *bolA* was induced at the start of this phase of growth. Overall results showed diverse classes of genes that were differentially expressed and related with different functions in the cell. However, only one *E. coli* sigma factor, the sigma E (σ^E), was changing. σ^E is known by the polymerase subunit responsible for the response to stress that harms the cellular envelope homeostasis regulating different genes involved in the synthesis of outer membrane proteins (OMPs). Interestingly, BolA was also described in previous works to be related with OmpC/OmpF ratio control. Thus, the role of BolA in σ^E expression was analyzed. In fact, σ^E was upregulated in the presence of a high amount of BolA. Moreover, it was also observed differential expression in genes that belong to the σ^E regulon which were also analyzed and some of them were significantly up or downregulated. Some of the RNAs identified and studied are non-coding small RNAs (sRNAs). These RNAs are usually related with stress response and rapid adaptation of bacteria to new environments. A relatively large percentage of the described sRNAs in the literature are related with regulation of OMPs expression. Up to now, of the many sRNAs discovered in *E. coli*, three are known to be σ^E-dependant. MicA, RybB and CyaR are the sRNAs that belong to the σ^E regulon. Two of them were detected to vary in the microarray experiment, RybB and CyaR, being upregulated. The increase levels of these sRNAs and σ^E clearly indicate an alteration of the envelope homeostasis and thus a cell response. These sRNAs impede the expression of different OMP proteins to avoid their translocation to the outer membrane and thus more destabilization in the cell envelope.

Other non-coding RNAs were noticeably regulated by BolA overexpression. Some still related with cell envelope protein regulation, OmrA, OmrB; other with regulation of iron storage proteins like RyhB; one without characterized function, RydB; and two related with transcription factors regulation and cell motility,

DsrA and CsrB. Of particular interest was CsrB sRNA, which is an antagonist of CsrA. It is described by sequestering this protein affecting its functions in the cell. CsrA protein has a fundamental role in carbohydrate's metabolism and translationally represses enzymes needed for normal cell motility. Since BolA is involved in biofilm formation, thus curli pathway synthesis and flagella inhibition, the regulation over CsrB can contribute for the understanding of the function of this transcription factor in the switch between motile and non-motile cells. For that, an interesting approach would be to analyse the master regulators of both flagella and curli synthesis pathways taking into account the presence or absence of BolA in the cell. A similar experiment could be performed in stationary phase, and then compare the results with exponential phase. The latest discoveries regarding small noncoding RNAs (sRNAs) in both pro- and eukaryotes have shown that the interaction of RNA with proteins and mRNAs plays a prominent role in the regulation of cellular processes. Taking into account our results and BolA involvement in non-coding RNA regulation, deep-sequencing analyses of a BolA mutant vs. wt would be of major interest to decipher the role of this new *E. coli* transcriptional regulator in the control of RNAs and their role in post-transcriptional control of gene expression.

Not just sRNAs are crucial for post-transcriptional control of gene expression. Ribonucleases (RNases) are the enzymes responsible for the processing and decay of RNA and their study brings important advances to the understanding of the regulation of gene expression. They have also been described as an important factor involved in the virulence mechanisms of several pathogenic organisms (Cheng et al., 1998; Erova et al., 2008; Tobe et al., 1992; Tsao et al., 2009), and the mode of action of these proteins is of major importance for virulence studies. In the final part of this Doctoral work I focused on the post-transcriptional regulation of gene expression by RNase R in the human pathogen *Streptococcus pneumoniae.* Until now, *S. pneumoniae* has only one hydrolytic

ribonuclease described that belongs to the RNase II-family of enzymes, the RNase R. This enzyme was already target of biochemical characterization (Domingues et al., 2009) but its role in the cell is still unknown. Downstream of *rnr* gene is located *smpB* coding sequence and upstream of *rnr* there is a *secG* open reading frame. Curiously, *smpB* 5' end overlaps with the 3' end of *rnr.* When this was observed for the first time, it raised the hypothesis of these two genes being expressed in an operon as a single transcriptional unit. In *E. coli,* RNase R associates with SmpB and the tmRNA in the ribosome rescue system and participates in the degradation of the mRNA on which tmRNA-dependent ribosome rescue occurs (Karzai and Sauer, 2001; Richards et al., 2006). Moreover, SmpB together with tmRNA, are the main components of the trans-translation system, a system important for quality control since it releases ribosomes stalled in non-stop transcripts and tags truncated proteins for their degradation by cellular proteases (Keiler, 2008; Richards et al., 2008). The overlapping of RNase R and SmpB in *S. pneumoniae* suggested some level of regulation between these two partners.

RNase R was previously described to be modulated in response to different stress conditions including cold-shock (Andrade et al., 2006; Cairrao et al., 2003; Chen and Deutscher, 2005). In fact we confirmed that in this human pathogen, RNase R mRNA and protein expression levels are also induced in cold shock response. Interestingly we pursued studies on the characterization of this enzyme and observed that it is indeed transcribed together in an operon encompassing *secG* and *smpB.* The observed genomic organization of *rnr* localized upstream of *smpB* was confirmed to be a common feature among Gram-positive bacteria. Moreover, we saw that the operon is under the control of a single promoter upstream of *secG.* It is the first time that *rnr* is showed to be co-transcribed with one of the major trans-translation players, which in this case is *smpB.* Processing of the operon to yield the mature gene products is likely to occur since different

putative cleavage sites were detected on the overlapping region of *rnr* with *smpB.* RNase R has been described as an important protein in the ribosome rescue system participating in the degradation of the mRNA on which tmRNA-dependent ribosome rescue occurs (Karzai and Sauer, 2001; Richards et al., 2006). In the study performed, a difference on the levels of *smpB* mRNA were noticed suggesting a role of RNase R in *smpB* degradation. Trans-translation is a SmpB dependent quality control mechanism in bacteria. For the first time we showed a direct involvement of RNase R in the modulation of *smpB* mRNA levels in *S. pneumoniae* with consequences on the SmpB protein available to the cell. This finding is of extreme importance since RNase R has also a role in the quality control of defective peptide synthesis being involved in the degradation of aberrant mRNAs that come out of the trans-translation mechanism. Knowledge of the cell control mechanisms may lead to greater understanding of virulence in *S. pneumoniae* and possibly the identification of new putative targets for virulence studies. For instance, is RNase R involved in the degradation of mRNAs coding to virulence factors in this bacterium? Or is it involved in the maturation of those mRNAs being itself essential for pathogenesis? As a future perspective would be interesting to analyse the role of this RNase R in the expression of capsular proteins involved in pathogenesis.

An additional future aim derived from this study is to characterize the enzymes involved in the processing of the RNase R operon. In the Gram-positive model bacteria *B. subtilis,* RNase J1 and J2 are two important RNases that are in the base of the model of RNA degradation pathways (Even et al., 2005; Mader et al., 2008). However, homologues of these enzymes were not described yet in *S. pneumoniae.* Until now, RNase P, RNase III, RNase Z, RNase M5, RNase H2 and RNase H3 are the known endonucleases described in this organism. Are those performing all the endonucleolytic tasks in the post-transcriptional regulation of

gene expression? Or are there RNase J1 and J2 like proteins in *S. pneumoniae* to help on this job?

The dogma of molecular biology postulates that DNA can be replicated to DNA, can be copied into mRNA (transcription) and proteins can be synthesized using the information in mRNA as a template (translation). With the recent year's discoveries, the dogma had to be redefined and nowadays RNA is accepted as a multifunctional molecule that besides having a fundamental role in the translation process, can also act as a regulator of gene expression. Maintenance of optimal levels of RNAs at any time and under any circumstance is an extremely difficult task to achieve and requires great coordination among all the factors involved in this control. It is also assumed that there is a cross-talk between transcription and degradation to maintain the balance that is best for the survival of microorganisms. With everyday advances in molecular biology field, the interest to know how cell vital mechanisms work is essential, thus the work presented in this dissertation constitutes an important step towards the comprehension of different transcriptional and post-transcriptional mechanisms acting on the regulation of bacterial gene expression.

References

Aldea, M., C. Hernandez-Chico, A.G.d.l. Campa, S.R. Kushner, and M. Vicente. 1988. Identification, Cloning, and Expression of bolA, an ftsZ-Dependent Morphogene of Escherichia coli. *Journal of Bacteriology*. 170:5169-5176.

Alyahya, S.A., R. Alexander, T. Costa, A.O. Henriques, T. Emonet, and C. Jacobs-Wagner. 2009. RodZ, a component of the bacterial core morphogenic apparatus. *Proc Natl Acad Sci U S A*. 106:1239-1244.

Andrade, J.M., F. Cairrao, and C.M. Arraiano. 2006. RNase R affects gene expression in stationary phase: regulation of ompA. *Mol Microbiol*. 60:219-228.

Barth, M., C. Marschall, A. Muffler, D. Fischer, and R. Hengge-Aronis. 1995. Role for the histone-like protein H-NS in growth phase-dependent and osmotic regulation of sigma S and many sigma S-dependent genes in Escherichia coli. *J Bacteriol*. 177:3455-3464.

Bendezu, F.O., C.A. Hale, T.G. Bernhardt, and P.A. de Boer. 2009. RodZ (YfgA) is required for proper assembly of the MreB actin cytoskeleton and cell shape in E. coli. *EMBO J*. 28:193-204.

Bertin, P., E. Terao, E.H. Lee, P. Lejeune, C. Colson, A. Danchin, and E. Collatz. 1994. The H-NS protein is involved in the biogenesis of flagella in Escherichia coli. *J Bacteriol*. 176:5537-5540.

Browning, D.F., and S.J. Busby. 2004. The regulation of bacterial transcription initiation. *Nat Rev Microbiol*. 2:57-65.

Cairrao, F., A. Cruz, H. Mori, and C.M. Arraiano. 2003. Cold shock induction of RNase R and its role in the maturation of the quality control mediator SsrA/tmRNA. *Mol Microbiol*. 50:1349-1360.

Chen, C., and M.P. Deutscher. 2005. Elevation of RNase R in response to multiple stress conditions. *J Biol Chem*. 280:34393-34396.

Cheng, Z.F., Y. Zuo, Z. Li, K.E. Rudd, and M.P. Deutscher. 1998. The vacB gene required for virulence in Shigella flexneri and Escherichia coli encodes the exoribonuclease RNase R. *J Biol Chem*. 273:14077-14080.

Domingues, S., R.G. Matos, F.P. Reis, A.M. Fialho, A. Barbas, and C.M. Arraiano. 2009. Biochemical characterization of the RNase II family of exoribonucleases from the human pathogens Salmonella typhimurium and Streptococcus pneumoniae. *Biochemistry*. 48:11848-11857.

Dorman, C.J. 2004. H-NS: a universal regulator for a dynamic genome. *Nat Rev Microbiol*. 2:391-400.

Erova, T.E., V.G. Kosykh, A.A. Fadl, J. Sha, A.J. Horneman, and A.K. Chopra. 2008. Cold shock exoribonuclease R (VacB) is involved in Aeromonas hydrophila pathogenesis. *J Bacteriol*. 190:3467-3474.

Even, S., O. Pellegrini, L. Zig, V. Labas, J. Vinh, D. Brechemmier-Baey, and H. Putzer. 2005. Ribonucleases J1 and J2: two novel endoribonucleases in *B.subtilis* with functional homology to *E.coli* RNase E. *Nucleic Acids Res*. 33:2141-2152.

Freire, P., R. Neves Moreira, and C.M. Arraiano. 2009. BolA inhibits cell elongation and regulates MreB expression levels. *J Mol Biol*. 385:1345-1351.

Freire, P., H.L. Vieira, A.R. Furtado, M.A. de Pedro, and C.M. Arraiano. 2006. Effect of the morphogene bolA on the permeability of the Escherichia coli outer membrane. *FEMS Microbiol Lett*. 260:106-111.

Guinote, I.B., Rute Gonçalves Matos, Patrick Freire, and Cecília Maria Arraiano. 2011. BolA Affects Cell Growth, and Binds to the Promoters of Penicillin-Binding Proteins 5 and 6 and Regulates Their Expression. *Journal of Microbiology and Biotechnology*. *in press*.

Haugen, S.P., W. Ross, and R.L. Gourse. 2008. Advances in bacterial promoter recognition and its control by factors that do not bind DNA. *Nat Rev Microbiol*. 6:507-519.

Jones, L.J., R. Carballido-Lopez, and J. Errington. 2001. Control of cell shape in bacteria: helical, actin-like filaments in Bacillus subtilis. *Cell*. 104:913-922.

Karzai, A.W., and R.T. Sauer. 2001. Protein factors associated with the SsrA.SmpB tagging and ribosome rescue complex. *Proc Natl Acad Sci U S A*. 98:3040-3044.

Kasai, T., M. Inoue, S. Koshiba, T. Yabuki, M. Aoki, E. Nunokawa, E. Seki, T. Matsuda, N. Matsuda, Y. Tomo, M. Shirouzu, T. Terada, N. Obayashi, H. Hamana, N. Shinya, A. Tatsuguchi, S. Yasuda, M. Yoshida, H. Hirota, Y. Matsuo, K. Tani, H. Suzuki, T. Arakawa, P. Carninci, J. Kawai, Y. Hayashizaki, T. Kigawa, and S. Yokoyama. 2004. Solution structure of a BolA-like protein from Mus musculus. *Protein Sci*. 13:545-548.

Keiler, K.C. 2008. Biology of trans-translation. *Annu Rev Microbiol*. 62:133-151.

Korn, E.D., M.F. Carlier, and D. Pantaloni. 1987. Actin polymerization and ATP hydrolysis. *Science*. 238:638-644.

Lang, B., N. Blot, E. Bouffartigues, M. Buckle, M. Geertz, C.O. Gualerzi, R. Mavathur, G. Muskhelishvili, C.L. Pon, S. Rimsky, S. Stella, M.M. Babu, and A. Travers. 2007. High-affinity DNA binding sites for H-NS provide a molecular basis for selective silencing within proteobacterial genomes. *Nucleic Acids Res*. 35:6330-6337.

Mader, U., L. Zig, J. Kretschmer, G. Homuth, and H. Putzer. 2008. mRNA processing by RNases J1 and J2 affects *Bacillus subtilis* gene expression on a global scale. *Mol Microbiol*. 70:183-196.

McLeod, S.M., and R.C. Johnson. 2001. Control of transcription by nucleoid proteins. *Curr Opin Microbiol*. 4:152-159.

Ohya, S., M. Yamazaki, S. Sugawara, S. Tamaki, and M. Matsuhashi. 1978. New cephamycin antibiotic, CS-1170: binding affinity to penicillin-binding proteins and inhibition of peptidoglycan cross-linking reactions in Escherichia coli. *Antimicrob Agents Chemother*. 14:780-785.

Richards, J., P. Mehta, and A.W. Karzai. 2006. RNase R degrades non-stop mRNAs selectively in an SmpB-tmRNA-dependent manner. *Mol Microbiol*. 62:1700-1712.

Richards, J., T. Sundermeier, A. Svetlanov, and A.W. Karzai. 2008. Quality control of bacterial mRNA decoding and decay. *Biochim Biophys Acta*. 1779:574-582.

Santos, J.M., P. Freire, M. Vicente, and C.M. Arraiano. 1999. The stationary-phase morphogene bolA from Escherichia coli is induced by stress during early stages of growth. *Mol Microbiol*. 32:789-798.

Santos, J.M., M. Lobo, A.P. Matos, M.A. De Pedro, and C.M. Arraiano. 2002. The gene bolA regulates dacA (PBP5), dacC (PBP6) and ampC (AmpC), promoting normal morphology in Escherichia coli. *Mol Microbiol*. 45:1729-1740.

Shiomi, D., M. Sakai, and H. Niki. 2008. Determination of bacterial rod shape by a novel cytoskeletal membrane protein. *EMBO J*. 27:3081-3091.

Tobe, T., C. Sasakawa, N. Okada, Y. Honma, and M. Yoshikawa. 1992. vacB, a novel chromosomal gene required for expression of virulence genes on the large plasmid of Shigella flexneri. *J Bacteriol*. 174:6359-6367.

Tsao, M.Y., T.L. Lin, P.F. Hsieh, and J.T. Wang. 2009. The 3'-to-5' exoribonuclease (encoded by HP1248) of Helicobacter pylori regulates motility and apoptosis-inducing genes. *J Bacteriol*. 191:2691-2702.

van den Ent, F., L.A. Amos, and J. Lowe. 2001. Prokaryotic origin of the actin cytoskeleton. *Nature*. 413:39-44.

Vieira, H.L., P. Freire, and C.M. Arraiano. 2004. Effect of Escherichia coli morphogene bolA on biofilms. *Appl Environ Microbiol*. 70:5682-5684.

White-Ziegler, C.A., and T.R. Davis. 2009. Genome-wide identification of H-NS-controlled, temperature-regulated genes in Escherichia coli K-12. *J Bacteriol*. 191:1106-1110.

Wood, T.K., A.F. Gonzalez Barrios, M. Herzberg, and J. Lee. 2006. Motility influences biofilm architecture in Escherichia coli. *Appl Microbiol Biotechnol*. 72:361-367.

Appendix

Publications

doi:10.1016/j.jmb.2008.12.026 *J. Mol. Biol.* (2008) **xx**, xxx–xxx

JMB

Available online at www.sciencedirect.com
ScienceDirect

ELSEVIER

COMMUNICATION

BolA Inhibits Cell Elongation and Regulates MreB Expression Levels

Patrick Freire, Ricardo Neves Moreira and Cecília Maria Arraiano*

Control of Gene Expression Laboratory, Instituto de Tecnologia Química e Biológica/Universidade Nova de Lisboa, Apartado 127, Av. da República, 2781-901 Oeiras, Portugal

Received 29 August 2008; received in revised form 5 December 2008; accepted 10 December 2008

The morphogene *bolA* is a general stress response gene in *Escherichia coli* that induces a round morphology when overexpressed. Results presented in this report show that increased BolA levels can inhibit cell elongation mechanisms. MreB polymerization is crucial for the bacterial cell cytoskeleton, and this protein is essential for the maintenance of a cellular rod shape. In this report, we demonstrate that *bolA* overexpression affects the architecture of MreB filaments. An increase in BolA leads to a significant reduction in MreB protein levels and *mreB* transcripts. BolA affects the *mreBCD* operon *in vivo* at the level of transcription. Furthermore, our results show that BolA is a new transcriptional repressor of MreB. The alterations in cell morphology induced by *bolA* seem to be mediated by a complex pathway that integrates PBP5, PBP6, MreB, and probably other regulators of cell morphology/elongation.

Edited by J. Karn

Keywords: BolA; MreB; transcriptional repressor; morphology; cytoskeleton

Penicillin-binding proteins (PBPs) are key players in cell elongation and division mechanisms.[1] In *Escherichia coli*, PBP2 is responsible for lateral murein extension, leading to cell elongation, while PBP3 is specific for septal murein production during cell division. Specific inhibition of PBP2 by mecillinam causes *E. coli* to grow as spherical cells, while inhibition of PBP3 using aztreonam blocks septal peptidoglycan synthesis, leading to a characteristic filamented cell phenotype.[1] PBP2 is essential for cell elongation. MreB is a structural homolog of actin that is also essential for cell elongation and maintenance of a bacterial rod shape.[2,3]

The morphogene *bolA* induces a spherical shape when overexpressed, and it has been established as a general stress response gene.[4] The expression of *bolA* is tightly controlled.[5–7] *bolA* increases biofilm formation[8] and modulates cell permeability;[9] in addition, it was demonstrated that *bolA* increases the expression of PBP5, PBP6 and *ampC* mRNA.[10,11] In this work, we analyzed the effect of BolA on cell growth and elongation using a set of specific antibiotics that induce known morphology alterations through the inhibition of PBPs. Results show that BolA inhibits the mechanism of cell elongation and can act as a new transcriptional repressor of MreB expression.

BolA expression affects growth rate and cell elongation

Plasmid pPFA02 was constructed by cloning the *bolA* coding region in-frame with a $(His)_6$ tag at the 5′ end in a pET28a plasmid under the control of a LacZ promoter (Novagen). CMA50 is a BL21(DE3) strain (Novagen) transformed with pPFA02 plasmid. High expression of $(His)_6$–BolA was achieved 30 min after 1 mM IPTG (Merck) was added to the growth medium. Microscopic phase-contrast observations showed that all cells became round or olive-shaped after 1 h of induction (Supplementary Fig. S1), demonstrating that overexpression of $(His)_6$–BolA induces the same cellular morphology alterations that are observed when the native BolA protein is overexpressed.[4] Two hours after induction of BolA, the optical density at 620 nm (OD_{620}) of the culture increased 2.5×; without induction of *bolA*, the OD_{620} increased 7× (data not shown). The overexpression of BolA appears to be sufficient to retard cell growth rate. Aztreonam is a specific inhibitor of PBP3 activity that prevents septation and induces

*Corresponding author. E-mail address: cecilia@itqb.unl.pt.

Abbreviations used: PBP, penicillin-binding protein; EF, elongation factor; *orf*, open-reading frame.

0022-2836/$ - see front matter © 2008 Published by Elsevier Ltd.

the formation of cellular filaments.[1] Exponentially growing cells were regular rod-shaped bacteria, but some filamenting cells were also visible (about 2% of the total cell population) (Fig. 1a). Addition of aztreonam induced cell filamentation, as expected (Fig. 1a1 and a2). When *bolA* expression was induced after aztreonam addition, cells remained shaped as filaments (Fig. 1b1 and b2). However, these filaments no longer increased in length. Elongation seemed to be arrested. Surprisingly, after 90 min of *bolA* overexpression, a branched phenotype arose (Fig. 1b2). However, when *bolA* was induced in exponential phase and aztreonam was added 30 min later, cells no longer became filaments as could be expected due to aztreonam effects and furthermore acquired a shorter morphology (Fig. 1c1 and c2). Even though some longer cells were still detected 30 min after aztreonam addition (Fig. 1c1), the population eventually reached 100% of short cells (Fig. 1c2). The levels of $(His)_6$–BolA in all the conditions observed by microscopy were determined by quantitative Western blot (Supplementary Fig. S2). Detection was performed with an anti-(His) antibody from GE Healthcare at a concentration of 1:5000. The intensity of the bands measured normalized by measurements of elongation factor (EF)-Tu protein as internal control. The levels of BolA were maintained with slight variations throughout all conditions. This ensured that BolA is produced in considerable levels whenever it is induced. Conversely, when no induction with IPTG was performed, no level of the protein was detected. Thus, BolA overexpression can prevent elongation of the cell. The absence of *bolA*-mediated

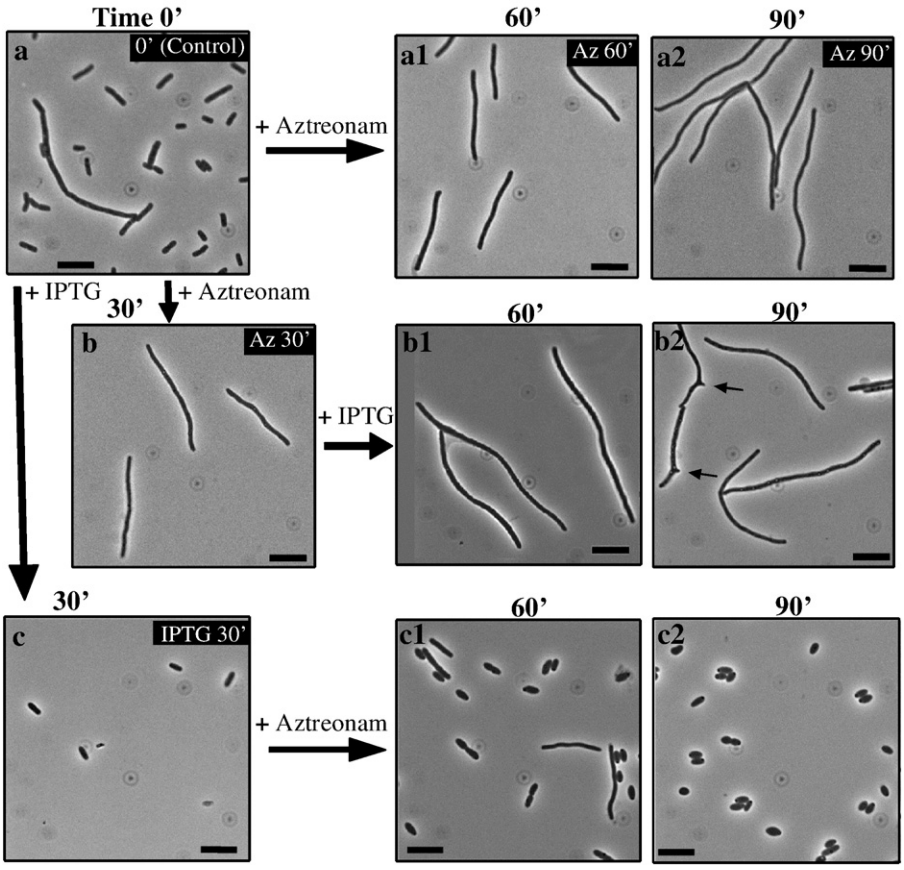

Fig. 1. Phase-contrast microscopy photographs. Batch cultures grown aerobically in LB medium at 37 °C and 120 rpm were launched from overnight cultures, diluted to an OD_{620} of 0.08.[4] Cells were harvested and fixed onto slides coated with a 1% agarose film.[12] Images were obtained using a DMRB microscope (Leica) under phase-contrast optics coupled to a CCD camera, with Leica software. CMA50 strain morphology alterations were observed in LB medium after addition of aztreonam (20 μg/mL) or IPTG (1 mM) starting at an OD_{620} of 0.4. (a) Exponentially growing CMA50 in LB medium. Time 0′ for aztreonam (Az) addition or IPTG addition. (a1) Sixty minutes after aztreonam addition to the medium. (a2) Ninety minutes after aztreonam addition. (b) Addition of IPTG to induce *bolA* expression 30 min after aztreonam treatment. (b1) Sixty minutes after aztreonam treatment and 30 min after *bolA* induction. (b2) Ninety minutes after aztreonam addition and 60 min after *bolA* induction. Black arrows show the beginning of cell branching. (c) Thirty minutes after induction of *bolA* by IPTG; time 0′ for addition of aztreonam. (c1) Sixty minutes after *bolA* induction and 30 min after aztreonam treatment. (c2) Ninety minutes after *bolA* induction and 60 min of aztreonam addition. Glucose (0.4% w/v) was added to the medium in control experiments to ensure complete shutdown of the expression of pPFA02 (data not shown). The levels of $(His)_6$–BolA were determined by quantitative Western blot and are supplied as supplementary data (Fig. S2). Black bar represents 5 μm.

Please cite this article as: Freire, P. *et al.*, BolA Inhibits Cell Elongation and Regulates MreB Expression Levels, *J. Mol. Biol.* (2008), doi:10.1016/j.jmb.2008.12.026

morphology when septation is inhibited before *bolA* induction (Fig. 1b1 and b2) indicates two possibilities: (a) the cells might be committed to an irreversible morphological pathway by the influence of aztreonam and BolA is no longer able to induce a rounder shape or (b) BolA-dependent inhibition of elongation might require a functional septation machinery, here inhibited by blocking PBP3, at least in an initial phase. Furthermore, the longer cells observed in Fig. 1c1 cannot become shorter in Fig. 1c2 by dividing since septation is inhibited. Therefore, the longer cells either might have been dying by lysis or were somehow being shortened by the overexpression of *bolA*.

BolA prevents cellular elongation/rod shape maintenance mechanisms

Cefmetazole is a cephalosporin that inhibits all *E. coli* PBPs except PBP2.[13] This antibiotic was used simultaneously with aztreonam in a similar experiment as in Fig. 1 to inhibit all PBP functions, except for PBP2, and focus the analysis of the effect of *bolA* on elongation mechanisms, independently of PBP5 or PBP6, previously shown to be regulated by BolA.[11] The results were generally the same as those illustrated in Fig. 1, showing that *bolA* overexpression is unable to revert the filament morphology when septation is blocked before its own induction (Fig. 2d1 and d2) and that cells are unable to elongate when *bolA* is overexpressed prior to septation inhibition (Fig. 2e1 and e2). However, the elongation now observed in Fig. 2d1 and d2 is strictly related to PBP2 activity, among all PBPs. It is interesting to verify that *bolA* can affect morphology independently of PBP5 and/or PBP6. Therefore, BolA overexpression either blocks PBP2-dependent cell elongation or affects another mechanism involved in the normal maintenance of the rod shape and essential for elongation. Overexpression of PBP2 was shown to be unable to revert the round phenotype caused by *bolA* overexpression back to a *bacilli* shape.[10] BolA might then be affecting other elements involved in cell elongation mechanisms that indirectly impair PBP2-dependent cell elongation. A good candidate is MreB, a structural homolog of actin essential for cell elongation and maintenance of the rod shape.[2,3] A possible correlation can also be established between *bolA*-induced round morphology and the spherical cells caused by mutations of the *mreB* gene or specific inhibition of MreB.[3]

BolA affects the architecture of MreB filaments

Immunofluorescence experiments to detect MreB filaments were performed in order to check for any influence of *bolA* on their spatial arrangement (Fig. 3.1). MreB polymerizes to form a spiraled structure along the interior of the cell wall.[2] MreB polymers forming the cytoskeleton were clearly visible when BolA was not overexpressed (Fig. 3.1a–d). When the expression levels of BolA increased, no more MreB filaments can be detected and the signal was spread all over the spherical cell (Fig. 3.1h–j). MreB fila-

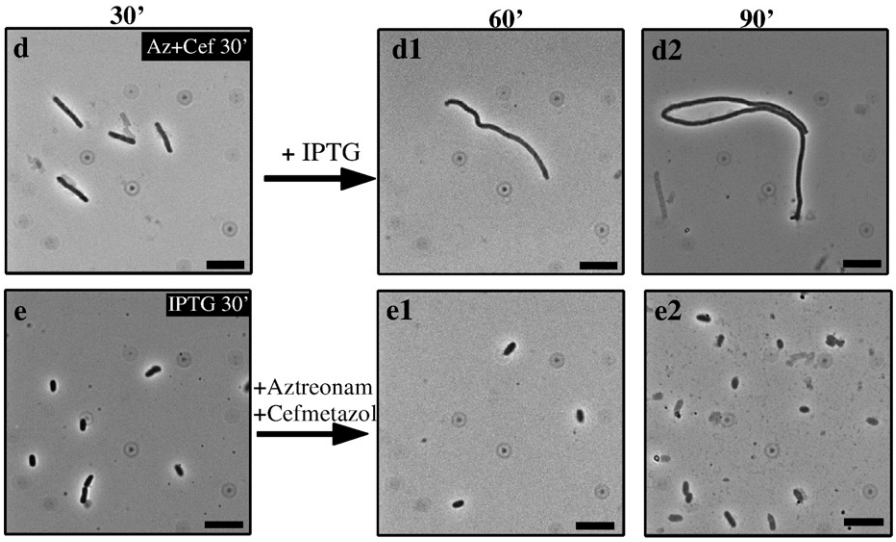

Fig. 2. Phase-contrast microscopy photographs. CMA50 strain morphology alterations were observed in LB medium after addition of aztreonam (20 μg/mL) plus cefmetazole (1 μg/mL) (cef) or IPTG starting at an OD_{620} of 0.4. Time 0′ of the experiment corresponds to the photo in Fig.1a. (d) Thirty minutes after aztreonam+cefmetazole addition to the medium; time 0′ for addition of IPTG to induce *bolA* expression. (d1) Sixty minutes after aztreonam+cefmetazole treatment and 30 min after *bolA* induction. (d2) Ninety minutes after aztreonam+cefmetazole addition and 60 min after *bolA* induction. (e) Thirty minutes after induction of *bolA* by IPTG; time 0′ for addition of aztreonam+cefmetazole. (e1) Sixty minutes after *bolA* induction and 30 min after aztreonam+cefmetazole treatment. (e2) Ninety minutes after *bolA* induction and 60 min after aztreonam+cefmetazole addition. The levels of $(His)_6$–BolA were determined by quantitative Western blot and are supplied as supplementary data (Fig. S2). Black bar represents 5 μm.

Please cite this article as: Freire, P. *et al.*, BolA Inhibits Cell Elongation and Regulates MreB Expression Levels, *J. Mol. Biol.* (2008), doi:10.1016/j.jmb.2008.12.026

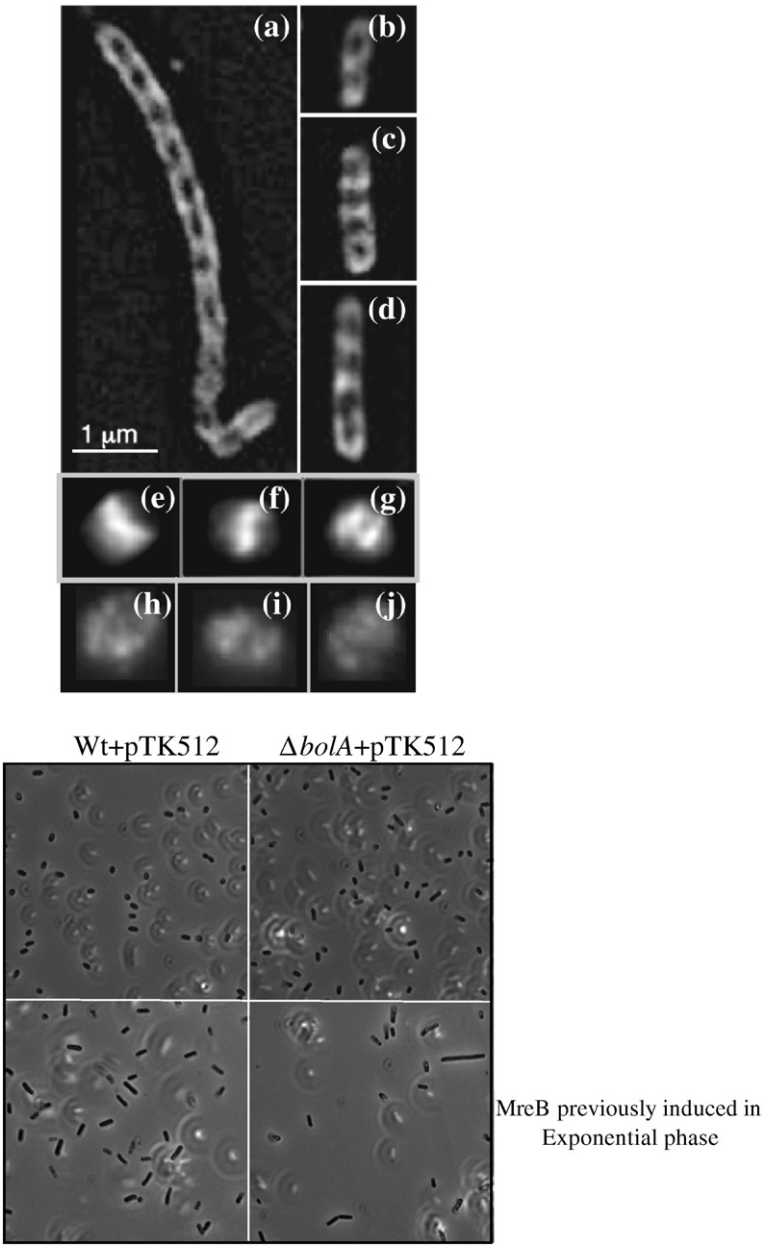

Fig. 3. (1) Analysis of *E. coli* cytoskeleton by immunofluorescence microscopy. Anti-MreB antibodies[14] were used at a 1:100 dilution; secondary TRITC anti-rabbit (Sigma), at 1:300. Cells were fixed in phosphate-buffered saline 1×, 4% formaldehyde, and 0.02% glutaraldehyde.[15] Cells were permeabilized with lysozyme (10 mg mL^{-1}) and applied to polylysine pretreated coverslips, fixed with methanol and acetone, and blocked with 2% bovine serum albumin and 0.05% Tween-20 in phosphate-buffered saline 1×. Images collected by immunofluorescence in a DMRB microscope (Leica) were treated with a deconvolution filter from MetaMORPH software. (a–d) Exponentially growing CMA50 cells showing the helical structures corresponding to the polymers of MreB forming the cytoskeleton. Subpanel (a) corresponds to an example of the filaments observed in these conditions. (e–g) Control experiment showing the visualization of MreB-defined filaments in round cells treated with mecillinam. (h–j) Overexpression of BolA 2 h after IPTG addition. Control experiments rule out any influence of glucose or IPTG on the morphology alterations (data not shown). The levels of $(His)_6$–BolA were determined by quantitative Western blot and are supplied as supplementary data (Fig. S2). (2) Phase-contrast microscopy photographs. Batch cultures grown aerobically in LB medium at 37 °C and 120 rpm were launched from overnight cultures, diluted to an OD_{620} of 0.08.[4] Cells were harvested and fixed onto slides coated with a 1% agarose film.[12] Images were obtained using a DMRB microscope (Leica) under phase-contrast optics coupled to a CCD camera, with Leica software. MG1693 and an isogenic Δ*bolA* were transformed with pTK512[14] plasmid that overexpresses the *mreBCD* operon with IPTG. The upper panel shows their morphology in the stationary phase of growth without induction. The lower panel shows the morphology alterations observed in the stationary phase when *mreBCD* is induced previously in the exponential phase of growth.

ments nevertheless remained detectable by immunofluorescence in round cells caused by addition of mecillinam, an inhibitor of PBP2 (Fig. 3.1e–g), showing that loss of MreB localization under *bolA* overexpression is not due to the shape alteration from rod to sphere. A similar observation was made when MreB polymerization was inhibited by A22, a specific inhibitor of MreB.[16] Therefore, the results obtained show that BolA overexpression affects MreB filaments' spatial organization.

MreB was induced with IPTG from plasmid pTK512[14] in several conditions and strains (data not shown) in an attempt to rescue the *bolA* spherical morphology. No reversion of cell morphology could be detected. BL21+pPFA02 strain was co-transformed with pTK512 to further study these effects. MreB and $(His)_6$–BolA were induced simultaneously with 1 mM IPTG. Overexpression of BolA and MreB together gave a mixed cell phenotype (round, lemon-shaped cells and rods), but, in general, longer cells were obtained due to the opposite effect of MreB in cell morphology (Supplementary Fig. S1). After 2 h of induction, when only BolA was overexpressed in the BL21+pPFA02 strain, cells became spherical or lemon-shaped. Non-induced cells presented the typical phenotype of BL21: rods with

Please cite this article as: Freire, P. *et al.*, BolA Inhibits Cell Elongation and Regulates MreB Expression Levels, *J. Mol. Biol.* (2008), doi:10.1016/j.jmb.2008.12.026

some filaments. MreB overexpression seems to reduce the impact of BolA in cell morphology. MG1693 and an isogenic *bolA* deletant were also transformed with pTK512 and studied in stationary phase to assess this effect on other strains. As above, after a rounder morphology was established, the induction of MreB was unable to restore longer cells. However, when MreB was induced in the exponential phase, it prevented the formation of shorter cells observed in the stationary phase. This effect is even more visible in *bolA* deletant strain, showing that MreB has an effect opposite to the influence of BolA in cell morphology (Fig. 3.2). Even though MreB overexpression cannot revert the morphology induced by BolA, higher levels of MreB in the cell clearly impair the induction of a rounder/shorter shape by BolA.

BolA affects MreB expression levels

The process of MreB filament formation is probably dependent on the intracellular levels of MreB, in a way similar to what happens in the actin polymerization process.[17] Therefore, if *bolA* significantly lowers the expression levels of MreB, this could be interfering with the architecture of MreB polymers. Western blots were performed to assess variations of MreB protein levels related to *bolA* overexpression. The results show that MreB protein levels were reduced by threefold when BolA was overexpressed (Fig. 4a). The detection of EF-Tu by specific antibodies on the same membranes shows that the variations observed are not due to pleiotropic effects of IPTG or BolA overexpression. BolA is thus shown to act as a new negative regulator of MreB. By interfering with the levels of MreB, *bolA* impairs the stability of the cytoskeleton in *E. coli*. The disruption of the internal cell scaffold could furthermore explain how cells could eventually shorten from a longer rod shape (Fig. 1c1) to olive-shaped cells (Fig. 1c2) when septation is inhibited.

RNA dot-blot experiments were performed to check whether the change in MreB protein levels derives from a reduction in *mreB* mRNA levels (Fig. 4b). The results show that induction of BolA levels was indeed able to significantly reduce the levels of *mreB* transcripts in less than 60 min. Therefore, *bolA* represses the levels of *mreB* mRNA. The levels of induced BolA were determined by quantitative Western blot and are provided as supplementary data (Fig. S3). The results obtained were further confirmed by quantitative reverse-transcription PCR. A Transcriptor First Strand cDNA Synthesis Kit (Roche) was used to reverse transcribe total RNA to cDNA using the random hexamer primer following the manual protocol. Quantitative PCRs were performed to amplify *mreB* and 16S RNA cDNAs using the primers RT-MREB (5′-acttgtccattgacctgggtactg-3′) and RT-MREB2 (5′-gccgccgtgcatgtcgatcatttc-3′) and the primers 16S rRNA F (5′-aga gtt tga tcc tgg ctc ag-3′) and 16S rRNA R (5′-acg gct acc ttg tta cga ctt-3′), respectively. Equal amounts of the sample were

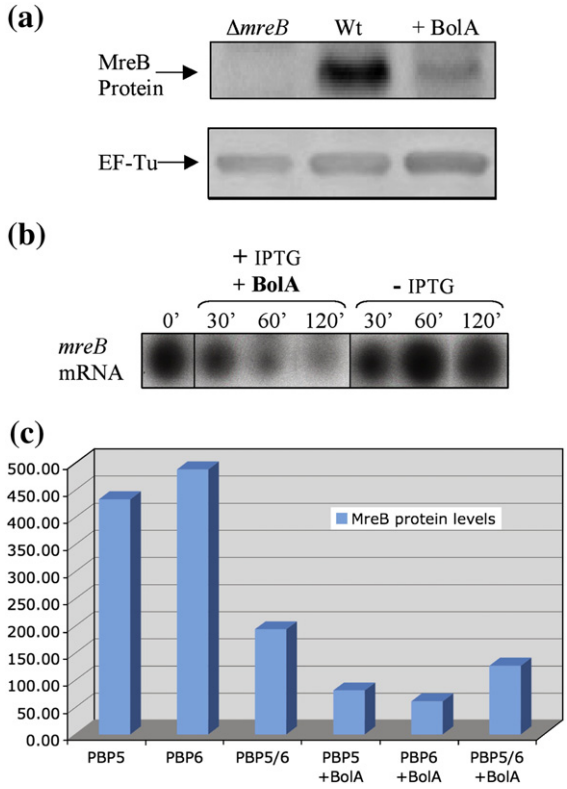

Fig. 4. (a) Western blot showing the levels of MreB in the cell. Bacterial proteins were extracted using Bugbuster (Novagen). Quantification was according to the Lowry method, and equal amounts of total protein were loaded in 12% SDS-PAGE gels.[18] Equal amounts of total protein extract of each sample were loaded in SDS-PAGE gels. After transfer, membranes were incubated with anti-MreB antibodies at a dilution of 1:10,000.[14] MC1000Δ*mreB* strain was used as negative control.[14] The top lane is Δ*mreB*, the middle lane shows the levels of this protein with basal expression of BolA, and the bottom lane shows the levels of MreB upon overexpression of BolA. EF-Tu detection was used as a control of total protein quantification. (b) Representative dot blot showing the analysis of steady-state *mreB* mRNA levels in CMA50 strain after overexpression of *bolA*. Total RNA was extracted as previously described.[7] Equal amounts of total RNA were blotted onto Hybond$^+$ membranes (GE) and fixed by UV light. An *mreB* DNA probe spanning the entire *mreB orf* was obtained by PCR using Taq polymerase (Roche) and the primers MreB1 (5′-attgacctgggtactgcg-3′) and MreB2 (5′-ctcttcgctgaacaggtc-5′) produced by STABVida. Membranes were hybridized and washed as described previously.[19] Membranes were autoradiographed using Biomax MR from Kodak, and bands were quantified with an IMAGEQUANT™ densitometer (Molecular Dynamics). 0′ represents *mreB* mRNA levels at an OD_{620} of 0.4. The levels of $(His)_6$–BolA were determined by quantitative Western blot and are supplied as supplementary data (Fig. S3). (c) MreB protein levels in PBP5, PBP6, and double PBP5/PBP6 mutants[11] in LB medium stationary phase. Quantifications were done by Western blot and normalized by EF-Tu determination on the same membranes. Lanes 1–3 show the results for the strains without *bolA* overexpression, while lanes 4–6 show MreB levels in the same conditions but with overexpression of *bolA* using plasmid pMAK580 (containing *bolA* with native promoters) as described previously.[11]

Please cite this article as: Freire, P. *et al.*, BolA Inhibits Cell Elongation and Regulates MreB Expression Levels, *J. Mol. Biol.* (2008), doi:10.1016/j.jmb.2008.12.026

loaded in 0.7% agarose gel (Supplementary Fig. S4). Quantification was done with ImageJ software and normalized by 16S RNA cDNA levels. The results obtained from three replicated experiments indicate intensities of 0.82±0.12 for the *mreB* cDNA band 60 min after induction of BolA and 1.45±0.22 without any induction. BolA therefore reduces the levels of *mreB* RNA to about 55% of their normal levels.

BolA represses operon *mreBCD* transcription by direct binding to its promoters

A plasmid pRMA1 was constructed containing the *gfp* gene encoding green fluorescent protein under the control of the promoters of the *mreBCD* operon using vector p363.[20] Total protein was extracted as above, with and without overexpression of BolA, and green fluorescent protein fluorescence was quantified in a Varian-Eclipse fluorescence spectrophotometer. The data obtained were normalized per cell by quantifying EF-Tu protein present in the different protein extracts. The fluorescence per cell (+BolA)/fluorescence per cell (Wt) ratio, representing the variations in *mreBCD* transcription by overexpression of *bolA*, was determined in BL21+ pPFA02 strain after 1 h of induction. The average ratio obtained was 0.64±0.04. BolA overexpression is therefore able to shut down transcription of *mreBCD* operon *in vivo* to about 64% of its normal expression, in correlation with the levels of *mreB* RNA detected above.

$(His)_6$–BolA was purified by histidine affinity chromatography using HiTrap chelating HP columns and an AKTA HPLC system (GE Healthcare). Purity of the protein was verified by SDS-PAGE. The purified protein was immobilized by amine coupling in a CM5 sensor chip on a Biacore 2000 system (GE Healthcare) following the manufacturer's instructions and analyzed by surface plasmon resonance. Biosensor assays were run at 25 °C in buffer containing 20 mM Tris–HCl, pH 8, 100 mM KCl, 1 mM dithiothreitol, and 25 mM ethylenediaminetetraacetic acid. Operon *mreBCD* promoters and *bolA* open-reading frame (*orf*) DNA encoding fragments were amplified by PCR using the primers mreB1 (5′-gccacttgatactaacgtg-3′) and mreB2 (5′-ggggcggaaaagaaaatc-3′) and the primers bolAX2 (5′-gtcacaatgtcccagccg-3′) and bolAX9 (5′-ccagacaaaacaaaacggcccg-3′), respectively. The amplified DNA fragments were injected as ligands. All experiments included replicate injections of six concentrations of each DNA sequence (ranging from 0 to 3 pM). Dissociation constants (K_d's) were calculated using the BIA Evaluation 3.0 software package, according to the fitting model 1:1 Langmuir binding. We determined a K_d of 6.9±2.4 nM for BolA interaction with *mreB* promoters and a K_d of 23.6±5.4 nM for the interaction with the *bolA orf*. The K_d of $(His)_6$–BolA interaction with the *bolA orf* sequence is therefore 3.5-fold higher than that with the *mreBCD* promoter sequence, showing that BolA has a significantly higher affinity for the promoter sequence of *mreBCD*. BolA is thus able to bind directly with high affinity to the promoter sequence of *mreB* and therefore acts as a new transcriptional repressor of MreB expression levels.

MreB concentration in fast-growing cells reaches 40,000 molecules/cell; in slow-growing cells, it was estimated at 17,000 molecules/cell.[14] Inversely, *bolA* mRNA levels are low in fast-growing cells but increase by about 20-fold in slow-growing cells;[4] the regulation of BolA expression might therefore be connected to the differential expression of MreB during different growth phases.

BolA plays a central role in a morphogenetic pathway including PBP5, PBP6, and MreB

BolA induces the expression levels of PBP5 and PBP6 at the onset of the exponential phase.[11] BolA overexpression was also shown to be unable to promote a round morphology in a PBP5/PBP6 double mutant.[11] The inhibition of all PBPs except PBP2 in Fig. 2e1 and e2 shows that *bolA* is nevertheless able to induce a shorter olive-shaped morphology independently of PBP5 or PBP6. A similar reduction in cell length was also reported in the PBP5/PBP6 double mutant.[11] BolA's effect on cell morphology alterations thus seems to be based on the integration of a complex set of regulations. The levels of MreB protein were analyzed in the PBP5 and PBP6 single and double mutants that were previously studied.[11] Protein levels were normalized by quantification of EF-Tu levels (Fig. 4c). A general negative effect of *bolA* overexpression on the levels of MreB is clearly confirmed (columns 4 to 6), even though this effect is lower in the double PBP5/PBP6 mutant. MreB levels are lower in the single mutants as compared with the double mutant upon overexpression of *bolA*. This low concentration might not be enough to permit polymerization of MreB filaments, as seen in the data presented in Fig. 3. The conjugated effects of BolA in MreB, PBP5, and PBP6 thus contribute to induce the round morphology in PBP single mutants. Likewise, the absence of a round morphology induced by *bolA* in the PBP5/PBP6 double mutant could be correlated not only to the lack of PBP5 and PBP6 but also to the higher levels of MreB observed in that strain. Furthermore, since this strain shows lower levels of MreB than the single mutants, independently of *bolA* overexpression (column 3), expression of MreB might also be influenced by PBP5 and/or PBP6. BolA therefore seems to play a central role in a complex web of regulators of cell morphology/elongation that includes PBP5, PBP6, MreB, and probably other factors. The induction of PBP5 and PBP6 and the reduction in MreB levels by BolA overexpression converge to inhibit cell elongation and induce a rounder morphology.

This work shows that *bolA*-induced cell morphology alterations are mediated by a complex pathway that integrates PBP5, PBP6, and MreB. The finding that BolA can directly repress the transcription of *mreBCD* and lower the levels of MreB in the cell presents a broad impact on cellular features, such as

Please cite this article as: Freire, P. *et al.*, BolA Inhibits Cell Elongation and Regulates MreB Expression Levels, *J. Mol. Biol.* (2008), doi:10.1016/j.jmb.2008.12.026

morphology maintenance and elongation mechanisms, especially in stress conditions when *bolA* is induced. It is also a major step toward understanding the regulation of MreB expression, a protein responsible for the cytoskeleton, an essential architectural element of the bacterial cell. Further studies will be necessary to provide more insights on these novel regulation pathways and how the different elements involved influence one another.

Acknowledgements

This work was funded by Fundação para a Ciência e a Tecnologia (Portugal). P.F. and R.N.M. are recipients of fellowships from Fundação para a Ciência e a Tecnologia. We thank Kenn Gerdes and Miguel Vicente for their kind gifts of anti-MreB serum as well as plasmid pTK512 and anti-EF-Tu serum, respectively. We also thank Gerhard Miksch for the kind gift of plasmid p363.

Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2008.12.026

References

1. Spratt, B. G. & Pardee, A. B. (1975). Penicillin-binding proteins and the cell shape in *E. coli*. *Nature*, **254**, 516–517.
2. Jones, L. J., Carballido-Lopez, R. & Errington, J. (2001). Control of cell shape in bacteria: helical, actin-like filaments in *Bacillus subtilis*. *Cell*, **104**, 913–922.
3. Van den Ent, F., Amos, L. A. & Lowe, J. (2001). Prokaryotic origin of the actin cytoskeleton. *Nature*, **413**, 39–44.
4. Santos, J. M., Freire, P., Vicente, M. & Arraiano, C. M. (1999). The stationary-phase morphogene *bolA* from *Escherichia coli* is induced by stress during early stages of growth. *Mol. Microbiol.* **32**, 789–798.
5. Freire, P., Amaral, J. D., Santos, J. M. & Arraiano, C. M. (2006). Adaptation to carbon starvation: RNase III ensures normal expression levels of bolA1p mRNA and σ^S. *Biochimie*, **88**, 341–346.
6. Lange, R. & Hengge-Aronis, R. (1991). Growth phase-regulated expression of *bolA* and morphology of stationary-phase *Escherichia coli* cells are controlled by the novel sigma factor sigma S. *J. Bacteriol.* **173**, 4474–4481.
7. Santos, J. M., Freire, P., Mesquita, F. S., Mika, F., Hengge, R. & Arraiano, C. M. (2006). Poly(A)-polymerase I links transcription with mRNA degradation via sigmaS proteolysis. *Mol. Microbiol.* **60**, 177–188.
8. Freire, P., Vieira, H. L., Furtado, A. R., de Pedro, M. A. & Arraiano, C. M. (2006). Effect of the morphogene *bolA* on the permeability of the *Escherichia coli* outer membrane. *FEMS Microbiol. Lett.* **260**, 106–111.
9. Vieira, H. L., Freire, P. & Arraiano, C. M. (2004). Effect of *Escherichia coli* morphogene *bolA* on biofilms. *Appl. Environ. Microbiol.* **70**, 5682–5684.
10. Aldea, M., Hernandez-Chico, C., de la Campa, A. G., Kushner, S. R. & Vicente, M. (1988). Identification, cloning, and expression of *bolA*, an *ftsZ*-dependent morphogene of *Escherichia coli*. *J. Bacteriol.* **170**, 5169–5176.
11. Santos, J. M., Lobo, M., Matos, A. P., de Pedro, M. A. & Arraiano, C. M. (2002). The gene *bolA* regulates *dacA* (PBP5), *dacC* (PBP6) and *ampC* (AmpC), promoting normal morphology in *Escherichia coli*. *Mol. Microbiol.* **45**, 1729–1740.
12. Donachie, W. D., Begg, K. J. & Vicente, M. (1976). Cell length, cell growth and cell division. *Nature*, **264**, 328–333.
13. Ohya, S., Yamazaki, M., Sugawara, S., Tamaki, S. & Matsuhashi, M. (1978). New cephamycin antibiotic, CS-1170: binding affinity to penicillin-binding proteins and inhibition of peptidoglycan cross-linking reactions in *Escherichia coli*. *Antimicrob. Agents Chemother.* **14**, 780–785.
14. Kruse, T., Moller-Jensen, J., Lobner-Olesen, A. & Gerdes, K. (2003). Dysfunctional MreB inhibits chromosome segregation in *Escherichia coli*. *EMBO J.* **22**, 5283–5292.
15. Harry, E. J., Pogliano, K. & Losick, R. (1995). Use of immunofluorescence to visualize cell-specific gene expression during sporulation in *Bacillus subtilis*. *J. Bacteriol.* **177**, 3386–3393.
16. Karczmarek, A., Baselga, R. M., Alexeeva, S., Hansen, F. G., Vicente, M., Nanninga, N. & den Blaauwen, T. (2007). DNA and origin region segregation are not affected by the transition from rod to sphere after inhibition of *Escherichia coli* MreB by A22. *Mol. Microbiol.* **65**, 51–63.
17. Korn, E. D., Carlier, M. F. & Pantaloni, D. (1987). Actin polymerization and ATP hydrolysis. *Science*, **238**, 638–644.
18. Sambrook, J., Maniatis, T. & Fritsh, E. F. (1989). Molecular Cloning, A Laboratory Manual, 2nd edit. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
19. Miller, J. H. (1992). Experiments in Molecular Genetics Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
20. Miksch, G. & Dobrowolski, P. (1995). Growth phase-dependent induction of stationary-phase promoters of *Escherichia coli* in different Gram-negative bacteria. *J. Bacteriol.* **177**, 5374–5378.

Please cite this article as: Freire, P. *et al.*, BolA Inhibits Cell Elongation and Regulates MreB Expression Levels, *J. Mol. Biol.* (2008), doi:10.1016/j.jmb.2008.12.026

REVIEW ARTICLE

The critical role of RNA processing and degradation in the control of gene expression

Cecília M. Arraiano, José M. Andrade, Susana Domingues, Inês B. Guinote, Michal Malecki, Rute G. Matos, Ricardo N. Moreira, Vânia Pobre, Filipa P. Reis, Margarida Saramago, Inês J. Silva & Sandra C. Viegas

Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Apartado, Oeiras, Portugal

Correspondence: Cecília M. Arraiano, Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Apartado 127, 2781-901 Oeiras, Portugal. Tel.: +351 214 469 547; fax: +351 214 469 549; e-mail: cecilia@itqb.unl.pt

Received 30 April 2010; revised 18 June 2010; accepted 20 June 2010.
Final version published online 26 July 2010.

DOI:10.1111/j.1574-6976.2010.00242.x

Editor: William Margolin

Keywords
RNases; RNA; post-transcriptional control of gene expression.

Abstract

The continuous degradation and synthesis of prokaryotic mRNAs not only give rise to the metabolic changes that are required as cells grow and divide but also rapid adaptation to new environmental conditions. In bacteria, RNAs can be degraded by mechanisms that act independently, but in parallel, and that target different sites with different efficiencies. The accessibility of sites for degradation depends on several factors, including RNA higher-order structure, protection by translating ribosomes and polyadenylation status. Furthermore, RNA degradation mechanisms have shown to be determinant for the post-transcriptional control of gene expression. RNases mediate the processing, decay and quality control of RNA. RNases can be divided into endonucleases that cleave the RNA internally or exonucleases that cleave the RNA from one of the extremities. Just in *Escherichia coli* there are $>$ 20 different RNases. RNase E is a single-strand-specific endonuclease critical for mRNA decay in *E. coli*. The enzyme interacts with the exonuclease polynucleotide phosphorylase (PNPase), enolase and RNA helicase B (RhlB) to form the degradosome. However, in *Bacillus subtilis*, this enzyme is absent, but it has other main endonucleases such as RNase J1 and RNase III. RNase III cleaves double-stranded RNA and family members are involved in RNA interference in eukaryotes. RNase II family members are ubiquitous exonucleases, and in eukaryotes, they can act as the catalytic subunit of the exosome. RNases act in different pathways to execute the maturation of rRNAs and tRNAs, and intervene in the decay of many different mRNAs and small noncoding RNAs. In general, RNases act as a global regulatory network extremely important for the regulation of RNA levels.

Introduction

General outline

Many cellular mechanisms cannot be fully understood without a profound knowledge of the RNA metabolism. Protein production depends not only on the levels of mRNAs but also on other RNA species. The translation of mRNAs is mediated by tRNAs and rRNAs and functional RNAs also intervene in the regulation of gene expression. Synergies between the structure and function of RNAs contribute towards orchestrating their fundamental role in cell viability.

Bacterial mRNAs are rapidly degraded and this allows the microorganisms to rapidly adapt to changing environments. Even though transcription is quite important to determine steady-state levels, increasingly it is being established that the role of post-transcriptional control is critical in the regulation of gene expression. Analyzing RNA degradation in prokaryotes has been particularly difficult not only due to the coupling of transcription, translation and mRNA degradation but also because most mRNAs undergo a rapid exponential decay with an average of 1.3 min at 37 °C. The rRNAs and tRNAs are usually more stable, but in order to be functionally active, they have to be processed to the mature form. It has been shown that the levels of small noncoding RNAs (sRNAs) are also highly dependent on post-transcriptional events. The knowledge collected makes it clear how far our understanding of RNA degradation has come in the last few years and how much remains to be discovered about this important genetic regulatory process. Applications of this knowledge in medicine and biotechnology are underway.

FEMS MICROBIOLOGY REVIEWS

RNases are the enzymes that intervene in the processing, degradation and quality control of all types of RNAs. A limited number of RNases can exert a determinant level of control acting as a global regulatory network, monitoring and adapting the RNA levels to the cell needs. Many of them are essential, but others exhibit a functional overlap and are interchangeable. RNases can act alone or they can cooperate in RNA degradation complexes. During RNA degradation, they do not only act as 'molecular killers' eliminating RNA species. RNases act according to the requirements of growth in adaptation to the environment; they play an extremely important role in contributing to the recycling of ribonucleotides, and also carry out surveillance, destroying aberrant RNAs that would produce detrimental proteins.

Individual RNA species differ widely with respect to their stability. The rate of turnover has no relation to the length of the gene, the segments that decay more rapidly can be anywhere in the mRNA and the stability of the gene transcripts seems to be regulated by determinants localized to specific mRNA segments. Secondary structure features can also influence the degradation by RNases.

Several factors can intervene in the decay mechanism: the sequence/structure of RNAs can act as stabilizer or destabilizer elements to specific RNases; the presence of ribosomes during active translation can hide some RNA loci that are vulnerable to RNases; poly(A) stretches are the preferred substrate for several RNases – therefore, the addition of poly(A) tails can modulate the stability of full-length transcripts and degradation intermediates and accelerate the decay of defective stable RNAs; *trans*-acting factors can bind to the RNAs and expose or hide RNA sites that are preferential targets for RNases – for instance, the host factor Hfq is known to bind sRNAs and affect their turnover; and other factors such as helicases can act in *trans* and contribute to RNA degradation because they unwind RNA structures and can change their accessibility to RNases.

In this review, we will focus on RNA processing and degradation in *Escherichia coli*, but we will also provide comparative examples from many other microorganisms. Namely, we will include the description of enzymes that exist in *Bacillus subtilis* and are absent in *E. coli*, we will provide examples from archaea and we will also include a section that makes a parallel to what happens in yeast.

We will start by describing most of the known RNases, characterizing their structure and function and the regulation of their expression. They will be divided into endonucleases, which cleave the RNA internally, and exonucleases, which cleave the RNA from one of the extremities. After the characterization of RNases, we will focus on their protein complexes involved in decay mechanisms. Then we will focus on the 'RNases in action'. Examples will be provided regarding the processing and degradation of RNAs. We will describe the maturation of rRNAs and tRNAs, and characterize the decay of many different mRNAs and sRNAs. Finally, we will compare with what is known in eukaryotic microorganisms, namely yeast. A small degree of overlap is unavoidable between sections on related topics. This allows for each section to be read and understood as an independent unit.

This review is intended to be an exhaustive and updated overview of what is known on RNAs, RNases and the post-transcriptional control of gene expression in microorganisms. It is expected that it can be used as a reference to put in perspective the critical role of RNA processing and degradation as a major global regulatory network.

Endonucleases

RNase E

RNase E, encoded by the *rne* gene, was first identified by a temperature-sensitive mutation (*rne-3071*) (Apirion & Lassar, 1978) and was initially described as an activity required for the processing of the *E. coli* 9S rRNA gene (Ghora & Apirion, 1978). The *ams* (altered mRNA stability) locus was also identified by a temperature-sensitive mutation (*ams-1*) (Ono & Kuwano, 1980) and was shown to play an important role in *E. coli* RNA turnover (Ono & Kuwano, 1979). The combination of the Ams and RNase II ts-alleles plus deficiency in polynucleotide phosphorylase (PNPase) was shown to substantially increase the half-life of bulk mRNA, and specific messengers were highly stabilized in the *ams-1 rnb-500 pnp-7* mutant (Arraiano *et al.*, 1988). Later, it was shown that these two previously identified genes, *rne* and *ams*, were actually different mutant alleles of the same gene encoding RNase E (Mudd *et al.*, 1990; Babitzke & Kushner, 1991; Melefors & von Gabain, 1991; Taraseviciene *et al.*, 1991). This important endonuclease is essential for cell growth, and the inactivation of temperature-sensitive mutants impedes processing and prolongs the lifetime of bulk mRNA (Apirion & Lassar, 1978; Ono & Kuwano, 1979; Arraiano *et al.*, 1988; Mudd *et al.*, 1990; Babitzke & Kushner, 1991; Melefors & von Gabain, 1991; Taraseviciene *et al.*, 1991). It has been reported that RNase E plays a central role in the processing of precursors of the 5S rRNA gene (Apirion & Lassar, 1978; Misra & Apirion, 1979), the 16S rRNA gene (Li *et al.*, 1999b), tRNAs (Ow & Kushner, 2002), transfer mRNA (tmRNA) (Lin-Chao *et al.*, 1999) and the M1 RNA component of the RNase P ribozyme (Lundberg & Altman, 1995; Ko *et al.*, 2008). Homologues of RNase E have been identified in > 50 bacteria, archaea and plants (Lee & Cohen, 2003).

Escherichia coli RNase E is a 1061-residue enzyme composed of two distinct functional regions (Fig. 1a). The amino-terminal half forms the catalytic domain (residues 1–529) and is relatively conserved among prokaryotes (Marcaida *et al.*, 2006). The carboxy-terminal half of RNase

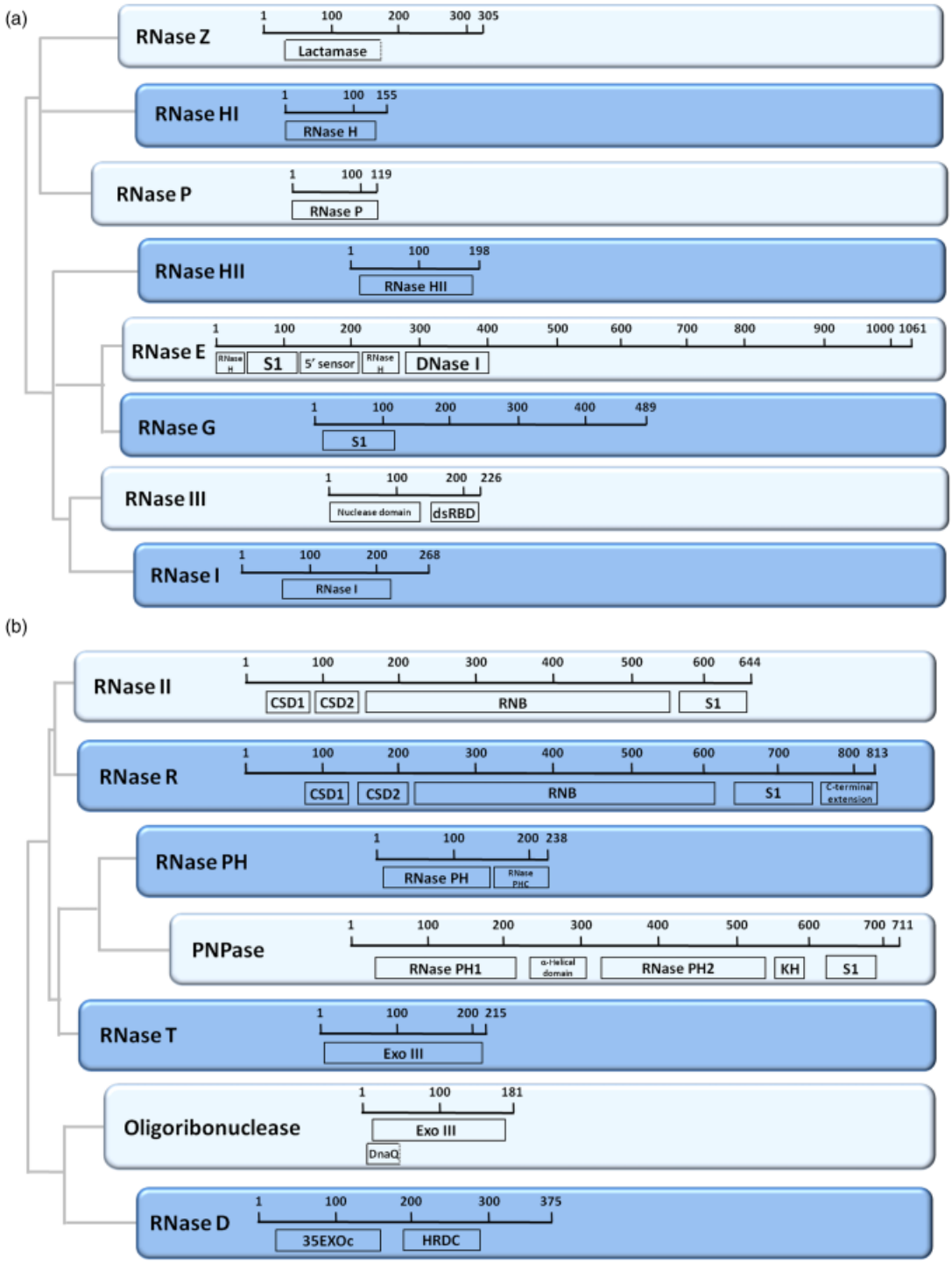

Fig. 1. Representative dendrograms of the endoribonucleases (a) and exoribonucleases (b) of *Escherichia coli*. This representation was based on the amino acid sequence of each enzyme, after a multiple alignment using the CLUSTAL program (Thompson *et al*., 1997). Near each enzyme is the length (number of amino acids) and architecture, emphasizing the domains of each enzyme. This representation was made based on the CDART program (Geer *et al*., 2002). These dendrograms were adapted from Barbas *et al*. (2006).

E (residues 530–1061) is a noncatalytic region, largely unstructured and poorly conserved (Callaghan *et al*., 2004). Segment A is located between residues 565 and 582 and is responsible for binding of RNase E to the inner cytoplasmic membrane (Khemici *et al*., 2008). Residues 601–700 form an arginine-rich segment that binds RNA *in vitro* and that is

believed to enhance the activity of RNase E in mRNA degradation *in vivo* (Lopez *et al.*, 1999; Ow *et al.*, 2000). Residues 701–1061 form a scaffold for interactions between RNase E and the other major components of the degradosome, a protein complex involved in mRNA decay (see Complexes of RNases) (Kaberdin *et al.*, 1998; Vanzo *et al.*, 1998).

The first crystal structure for a member of the RNase E family has been determined at 2.9 Å, and it reveals that the catalytic domain of RNase E forms a homotetramer with a molecular mass of roughly 260 kDa, organized as a dimer of dimers (Callaghan *et al.*, 2005a). Each protomer is composed of two globular portions: the 'large' and 'small' domains. The 'large' domain can be divided into four subdomains that closely resemble established folds. One is related to the RNase H endoribonuclease family, but is inactive. In this subdomain an S1 domain is embedded and has a fold that participates in the recognition of the 5′ terminus of RNA (5′-sensor). The rest of the large domain is similar to the repetitive structural element within the endodeoxyribonuclease DNase I. In isolation, each protomer appears elongated, with a large domain comprising the subdomains (S1, 5′-sensor, RNase H and DNase I), an elongated linker region (Zn-link) and then the small domain. The dimer–dimer interface is formed by the small domains. At the junction point, there is a zinc-binding site (Callaghan *et al.*, 2005a, b). The arrangement of the domains within each dimer resembles the blades and handles of an open pair of scissors.

Escherichia coli RNase E is a single-stranded, nonspecific endonuclease with a preference for cleaving A/U-rich sequences (Mackie, 1992; McDowall *et al.*, 1995). *In vitro* experiments have shown that purified *E. coli* RNase E prefers to cleave RNAs that are monophosphorylated at the 5′ end (Mackie, 1998). Recently, it was shown that RNA pyrophosphohydrolase (RppH) converts the 5′ terminus of primary transcripts from a triphosphate to a monophosphate (Celesnik *et al.*, 2007; Deana *et al.*, 2008). However, some structured substrates can be cleaved independent of its state of phosphorylation by RNase E even if the 5′ end forms a secondary structure (Baker & Mackie, 2003; Hankins *et al.*, 2007). This indicates that while the 5′-monophosphate-dependent pathway makes a significant contribution to mRNA degradation (Mackie, 1998, 2000), there is another pathway of initial substrate recognition by RNase E termed 'bypass' or 'internal entry' (Baker & Mackie, 2003; Kime *et al.*, 2009).

The crystal structure explains some features of the protein and suggests a mechanism of RNA recognition and cleavage. A pocket is formed between the 5′-sensor and the RNase H subdomains and can bind a monophosphate group at a 5′ end (Callaghan *et al.*, 2005a). The catalytic site is physically separated from the 5′-sensing site. It contains conserved residues on the surface of the DNase I subdomain of RNase E and coordinate a magnesium ion implicated in catalysis. A 'mouse-trap' model for communication between the 5′-sensing pocket and the site of catalysis has been suggested: S1- and 5′-sensing domains move together as one body to clamp down the substrate (Koslover *et al.*, 2008). This conformational change suggests a mechanism of RNA recognition and catalysis that explains the enzyme's preference for substrates with a 5′-monophosphate over a 5′-triphosphate and 5′-hydroxyl RNA. Substantial flexibility was also observed at one of the dimer–dimer interfaces, a deformation that may be essential to accommodate structured RNA for processing by internal entry.

The cellular level and activity of RNase E are subject to complex regulation. First, the enzyme concentration in the cell is regulated by a feedback loop in which RNase E modulates the decay of its own mRNA, maintaining the level of the enzyme within a narrow range (Mudd & Higgins, 1993; Jain & Belasco, 1995; Diwa *et al.*, 2000; Sousa *et al.*, 2001; Ow *et al.*, 2002). Second, the efficiency of RNase E cleavage depends on the structure of the substrates and the accessibility of putative cleavage sites. A 5′-monophosphate in substrate RNAs serves as an allosteric activator of RNase E activity (Mackie, 1998; Jiang & Belasco, 2004). Third, interactions of mRNA targets with Hfq and sRNAs play an important role in the cleavage of certain mRNAs by RNase E (Wagner *et al.*, 2002). Fourth, the activity of RNase E is globally affected by protein inhibitors, namely the L4 ribosomal protein, RraA and RraB (the regulator of RNase activity A and B, respectively) that interact with RNase E and inhibit RNase E endonucleolytic cleavages of a selective group of transcripts (Lee *et al.*, 2003; Gao *et al.*, 2006). Fifth, the membrane localization of RNase E and its association with the bacterial cytoskeleton may affect its function through various mechanisms (Liou *et al.*, 2001; Khemici *et al.*, 2008; Taghbalout & Rothfield, 2008).

Some variants of RNase E can be found in *Alphaproteobacteria*, *Synechocystis* spp. and in the high G+C Gram-positive bacteria (Condon & Putzer, 2002). In *Rhodobacter capsulatus*, RNase E is the enzyme responsible for the majority of the endonucleolytic cleavages. *Rhodobacter capsulatus* RNase E (118 kDa) has a conserved N-terminal region (Jäger *et al.*, 2001) and a C-terminal portion, probably involved in the scaffold of degradosome assembly. It was purified in two different complexes: one where it is associated with a helicase and an unidentified protein and the other, which was coupled with a helicase, Rho and an unidentified protein (Jäger *et al.*, 2001). Moreover, in *R. capsulatus*, this enzyme is involved in the endonucleolytic processing and stabilization of *cspA* mRNA (Jäger *et al.*, 2004). Similar to *R. capsulatus*, *Pseudomonas syringae*, a psychrophilic bacterium, also has an RNase E that is associated with RNase R and the DEAD-box helicase RhlE in a degradosome (see Complexes of RNases) (Purusharth *et al.*, 2005).

RNase G

Escherichia coli RNase G was initially identified by its role in chromosome segregation and cell division (Okada *et al.*, 1994). Overproduction of this protein led to morphological changes in which the bacteria formed anucleated chained cells containing long axial filaments, justifying its former name, *cafA* (cytoplasmic axial filament) (Okada *et al.*, 1994). RNase G was subsequently shown to exhibit endonuclease activity both *in vivo* (Li *et al.*, 1999b; Wachi *et al.*, 1999; Umitsuki *et al.*, 2001) and *in vitro* (Jiang *et al.*, 2000; Tock *et al.*, 2000). RNase G is a paralogue of RNase E (McDowall *et al.*, 1993), belonging to the RNase E/G family, and is also involved in the degradation and processing of RNA (Carpousis *et al.*, 2009).

A strong resemblance has been identified between RNase G and the amino-terminal portion of *E. coli* RNase E, sharing a high level of sequence identity (35%) and similarity (50%) (McDowall *et al.*, 1993) (Fig. 1a). Purified RNase G has *in vitro* properties similar to RNase E and both enzymes are required for a two-step sequential reaction of 5′ maturation of the 16S rRNA gene (Li *et al.*, 1999b; Wachi *et al.*, 1999). Their activity is 5′ end dependent and both RNases attack substrates in A+U-rich regions (Jiang *et al.*, 2000; Tock *et al.*, 2000). Moreover, residues of RNase E that can contact a 5′-monophosphorylated end and coordinate the catalytic magnesium ion are conserved in RNase G (McDowall *et al.*, 1993; Callaghan *et al.*, 2005a). RNase G seems to have a higher preference for 5′-monophosphorylated substrates than RNase E (Tock *et al.*, 2000) and the precise cleavage sites of RNase E and RNase G are not strictly conserved (Li *et al.*, 1999b; Tock *et al.*, 2000). The 5′-monophosphate end, which stimulates RNase G, is generated by RppH (Deana *et al.*, 2008) or by other endonucleases (Lee *et al.*, 2002).

Whereas cells lacking RNase E are normally nonviable (Apirion & Lassar, 1978; Ono & Kuwano, 1979), RNase G is dispensable for viability (Li *et al.*, 1999b; Wachi *et al.*, 1999) and is present in lower abundance (Lee *et al.*, 2002). Some functional homology between RNase G and RNase E was suggested by the observations that RNase G expression can confer viability to the *rne* deletion mutant strain (Lee *et al.*, 2002). However, at intracellular physiological levels, RNase G cannot complement RNase E mutations (Lee *et al.*, 2002; Ow *et al.*, 2003). Recently, single amino acid changes in the predicted RNase H domain of RNase G led to complementation of RNase E deletion mutants, suggesting that this region of the two proteins may help distinguish their *in vivo* biological activities (Chung *et al.*, 2010). However, these RNase G mutant proteins do not fully substitute RNase E in mRNA decay and tRNA processing (Chung *et al.*, 2010).

Microarray data showed that RNase G controls the level of transcripts associated with sugar metabolism centered on glycolysis (*adhE*, *pgi*, *glk*, *nagB*, *acs*, *eno*, *tpiA*) (Lee *et al.*, 2002), and it has been shown that strains defective in RNase G produce increased levels of pyruvic acid (Sakai *et al.*, 2007). These results suggest that RNase G is involved in the regulation of central metabolism.

RNase III

RNase III was originally identified by Robertson *et al.* (1968) in extracts of *E. coli* as the first specific double-stranded RNA (dsRNAs) endoribonuclease. Members of the RNase III family are widely distributed among prokaryotic and eukaryotic organisms, sharing structural and functional features (Lamontagne *et al.*, 2001) (Fig. 1a). However, until now, homologues of RNase III have not been found in the genomes of archaea (Condon & Putzer, 2002). All enzymes of this family are hydrolytic and have a specificity for dsRNAs, generating 5′-monophosphate and 3′-hydroxyl termini with a two-base overhang at the 3′ end (Meng & Nicholson, 2008). The RNase III family comprises four classes, according to their polypeptide structure. The class I is the simplest, containing an endonuclease domain (NucD), characterized in several bacteria by the presence of a highly conserved amino acid stretch NERLEFLGDS, and a dsRNA-binding domain (dsRBD) (Blaszczyk *et al.*, 2001). The class II is exemplified by the *Drosophila melanogaster* Drosha protein, which contains a long N-terminal extension, followed by two NucD and a single dsRBD. The class III is represented by Dicer, which has an N-terminal helicase/ATPase domain, followed by a domain of unknown function (DUF283), a centrally positioned Piwi Argonaute Zwille (PAZ) domain and a C-terminal configuration like Drosha, consisting of two NucD and one dsRBD (Drider & Condon, 2004; MacRae & Doudna, 2007). Finally, the class IV is only represented, to date, by the Mini-RNase III of *B. subtilis*, which is constituted by a single NucD domain (Redko *et al.*, 2008).

The class I members of the RNase III family are ubiquitously found in bacteria, bacteriophages and some fungi (MacRae & Doudna, 2007). *Escherichia coli* RNase III has served as the prototypical member of the family. In this model microorganism, RNase III is encoded by the *rnc* gene, and is active as a 52 kDa homodimer (Li & Nicholson, 1996). Each monomer contains a C-terminal dsRBD, located in the last 74 amino acids, which is responsible for substrate recognition and adopts a tertiary fold with the characteristic α_1-β_1-β_2-β_3-α_2 structure that is conserved throughout the RNase III family (Blaszczyk *et al.*, 2001). Additionally, each monomer contains an N-terminal NucD. When the two monomers are combined (RNase III homodimer), they form a single processing center in the subunit interface, in which each monomer contributes to the hydrolysis of one RNA strand of the duplex substrate. Ji and

colleagues (Blaszczyk *et al.*, 2004; Gan *et al.*, 2006) resolved the structure of the hyperthermophilic bacteria *Aquifex aeolicus* RNase III and the data have revealed two functional forms of dsRNA binding by RNase III: a catalytic form, functioning as a dsRNA-processing enzyme, cleaving both natural and synthetic dsRNA, and a noncatalytic form, in which RNase III plays the role of a dsRNA-binding protein (without cleaving). The latter activity is in agreement with previous studies in which this enzyme binds certain substrates in order to influence gene expression, affecting RNA structures (Court, 1993; Oppenheim *et al.*, 1993; Dasgupta *et al.*, 1998; Calin-Jageman & Nicholson, 2003). Furthermore, magnesium (Mg^{2+}) is the preferred cofactor. Recent data are indicative that each active site contains two divalent cations during substrate hydrolysis (Meng & Nicholson, 2008).

The RNase III substrate selection consists of a combination of structural determinants and sequence elements referred to as reactivity epitopes, such as the helix length, the strength of base-pairing or the occurrence of specific nucleotide pairs (termed proximal and distal boxes) located at defined positions related to the cleavage site. In addition, there are also two classes of double-helical elements that can function as negative determinants, which can either inhibit the recognition of this endoribonuclease or suppress the cleavage (without affecting recognition) (Zhang & Nicholson, 1997; Pertzev & Nicholson, 2006b).

RNase III in *E. coli* is not essential; however, it was observed that mutants for this endoribonuclease have a slow-growth phenotype (Nicholson, 1999). This enzyme was initially identified due to its role in the maturation of tRNA precursors and rRNA. Regarding the maturation of rRNA, RNase III is involved in the processing of 16S and 23S from a 30S rRNA gene precursor (Babitzke *et al.*, 1993). In *Salmonella* and other members of *Alphaproteobacteria*, RNase III is also responsible for the cleavage of the intervening sequences (IVS) found in their 23S rRNA gene (Evguenieva-Hackenberg & Klug, 2000), and is also involved in the decay of several mRNA species (Condon & Putzer, 2002; Calin-Jageman & Nicholson, 2003). For example, in *E. coli*, this enzyme participates in the first step of the decay of *pnp* mRNA (Régnier & Portier, 1986), the gene encoding PNPase, downregulating its synthesis (Régnier & Grunberg-Manago, 1990; Robert-Le Meur & Portier, 1992; Jarrige *et al.*, 2001). Interestingly, this endoribonuclease also has the ability to regulate its own synthesis with a specific cleavage near the 5′ end of its own mRNA that removes a stem loop, which acts as a degradation barrier (Bardwell *et al.*, 1989; Matsunaga *et al.*, 1996).

RNase III participates as a stress response modulator, controlling the steady-state levels of genes involved in cellular adaptation to stress (Santos *et al.*, 1997; Freire *et al.*, 2006; Sim *et al.*, 2010). It was seen in *Salmonella typhimurium* that RNase III regulates the levels of the sRNA MicA (Viegas *et al.*, 2007), a main regulator of the abundant outer membrane protein OmpA that plays an important structural role in the cell and is involved in pathogenesis (Guillier *et al.*, 2006). The enzyme is also involved in the decay of sRNA/mRNA complexes upon translational silencing (Vogel *et al.*, 2004; Afonyushkin *et al.*, 2005; Huntzinger *et al.*, 2005; Kaberdin & Blasi, 2006). In this way, cleavage by RNase III within the sRNA/mRNA duplex and the resulting subsequent decay of the mRNA intermediate by the *E. coli* RNA decay machinery could resemble the RNA interference (RNAi) in the eukaryotic cells (Agrawal *et al.*, 2003). RNAi is an evolutionarily conserved phenomenon that functions as a safeguard for the maintenance of genomic integrity. This phenomenon allows the selective post-transcriptional downregulation of target genes in the cells, in which RNase III-like enzymes dictate the degradation of dsRNA molecules (Jagannath & Wood, 2007; Ma *et al.*, 2007; Jinek & Doudna, 2009). Accordingly, the RNase III family has been associated with gene expression regulation, potential antivirus agents and tumor suppressors (Lamontagne *et al.*, 2001).

Bs-RNase III is a homologue of *E. coli* RNase III in *B. subtilis*. It is a 28-kDa protein (Mitra & Bechhofer, 1994), encoded by the *rncS* gene (Mitra & Bechhofer, 1994; Herskovitz & Bechhofer, 2000). In contrast to *E. coli* and *Staphylococcus aureus*, where the RNase III gene can be deleted without loss of viability, in *B. subtilis* and in the yeast, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, this enzyme is essential (Huntzinger *et al.*, 2005). Although the local environment of the site of Bs-RNase III cleavage appears to be very similar to that of *E. coli* RNase III, there are important differences in their substrate specificity (Mitra & Bechhofer, 1994; Wang & Bechhofer, 1997). Some of the substrates for this enzyme are the 30S ribosomal precursor RNA (Wang & Bechhofer, 1997) and the small cytoplasmic RNA (scRNA) (Oguro *et al.*, 1998; Yao *et al.*, 2007). More recently, another RNase III-like protein was identified in *B. subtilis* called Mini-III, reported to be involved in 23S rRNA gene maturation (Redko *et al.*, 2008). Interestingly, like Bs-RNaseIII, Mini-III does not seem to have endogenous mRNA substrates (Bechhofer, 2009). In *Lactococcus lactis*, RNase III is encoded by the *rnc* gene and plays a determinant role in the control of *citQRP* mRNA stability (Drider *et al.*, 1998, 1999). Complementation assays performed in *E. coli* showed that *L. lactis* RNase III can process *E. coli* rRNAs and regulate the levels of PNPase mRNA, substituting the endogenous RNase III (Amblar *et al.*, 2004).

Taken together, the functional and evolutionary conservation of the RNase III family in bacteria and higher organisms is indicative of their biological relevance in RNA maturation and degradation. Despite the fact that RNase E is

considered the major RNase that catalyzes the initial rate-determining cleavage of several transcripts, the RNase III family of enzymes has emerged as one of the most important groups of endoribonucleases in the control of RNA stability (Jaskiewicz & Filipowicz, 2008).

RNase H

Both RNase III and RNase H are representatives of components of the RNAi machinery and both are Mg^{2+}-dependent hydrolytic endoribonucleases. The analysis of the crystal structure of *E. coli* RNase H (Yang *et al.*, 1990) revealed the stepwise participation of two magnesium atoms in the enzyme mechanism (Nowotny & Yang, 2006).

RNases H are enzymes that cleave the RNA of RNA/DNA hybrids that are formed during replication and repair, preventing aberrant chromosome replication (for a review, see Condon & Putzer, 2002; Worrall & Luisi, 2007; Tadokoro & Kanaya, 2009). It is a ubiquitous enzyme distributed among all domains of life, and three different RNase H enzymes have been identified (HI, HII and HIII) (Ohtani *et al.*, 1999). In *E. coli*, 95% of RNase H activity is provided by RNase HI (widely distributed in *Proteobacteria*) and the remainder by RNase HII (Fig. 1a). In *B. subtilis*, RNase H activity is mostly provided by RNase HII and HIII. RNase H activity is essential to both bacteria. Thus, the inactivation of one of the *rnh* genes, but not both, is tolerated in these two organisms (Itaya *et al.*, 1999; Ohtani *et al.*, 1999).

RNase HII is widely distributed in bacteria and archaea, while RNase HIII is only present in a limited number of bacteria (Ohtani *et al.*, 1999). Proteins similar to HI and HII (named H1 and H2, respectively) can also be found in eukaryotes, but are larger and more complex than their prokaryotic counterparts (see Cerritelli & Crouch, 2009 for a review). The RNase H domain was also described in retroviruses (RNase HI), where it is associated with a reverse transcriptase (Davies *et al.*, 1991; Mian, 1997).

The PIWI domain of the eukaryotic Argonaute proteins, involved in RNA silencing, is structurally similar to the RNase H domain and conserves the residues necessary for RNase H endonucleolytic activity (Song *et al.*, 2004; Kitamura *et al.*, 2010). The eukaryotic Ago proteins showing endonuclease activity (slicer) can digest one RNA strand of the RNA/RNA hybrid. In contrast, the few prokaryotic Ago proteins known show a higher affinity for RNA/DNA hybrids. Very recently, it was reported for the first time that *Pyrococcus furiosus* RNase HII (*pf*-RNase HII) can digest an RNA/RNA hybrid in the presence of Mn^{2+} (Kitamura *et al.*, 2010).

RNase P

RNase P is a ribozyme considered to be a vestige from the 'RNA world'. It was discovered by Sidney Altman, almost 40 years ago (Robertson *et al.*, 1972), and for this, he received the Nobel Prize in Chemistry in 1989. This ancestral protein is a quasi-universal endoribonuclease found in all three domains of life: Bacteria, Eukarya (and eukaryotic organelles) and Archaea. RNase P is best known for universally catalyzing the endonucleolytic cleavage of the extra nucleotides in the 5′ end of the pre-tRNAs to generate the mature tRNAs (for a recent review by Sidney Altman, see Liu & Altman, 2009).

This ribozyme appears to have adapted to modern cellular life by adding protein to the RNA catalytic core. The bacterial version is the most simple, with a single RNA [350–400 nucleotide (nt), encoded by the *rpnB* gene] and a single small protein subunit (approximately 15 kDa, encoded by the *rpnA* gene) (Fig. 1a), both essential for cell viability (Shiraishi & Shimura, 1986; Kirsebom *et al.*, 1988; Baer *et al.*, 1989). In archaea and eukaryotes, the RNA subunit is bound by multiple proteins (at least four and nine proteins, respectively) with no relationship with their bacterial counterpart (Hall & Brown, 2002).

Five distinct structural classes of RNase P RNAs have been defined, based on the RNA secondary structure. In bacteria, two distinct types predominate: the A type (for ancestral), represented by *E. coli* RNase P RNA, and the B type (for *Bacillus*), confined to the low G+C Gram-positive bacteria (Chen *et al.*, 1998; Massire *et al.*, 1998; Smith *et al.*, 2007). Although evolution retained the catalysis function associated with the RNA subunit, the protein(s) play vital supporting roles. The higher protein : RNA mass ratio in the archaeal and eukaryal holoenzymes reflects a recruitment of protein cofactors during evolution, broadening the substrate spectrum in the more complex cellular environments (Liu & Altman, 1994).

In the bacterium *A. aeolicus*, candidate genes for *rpnA* and *rpnB* could not be identified (Willkomm *et al.*, 2002; Lombo & Kaberdin, 2008). However, recent work has demonstrated the existence of an RNase P-like activity in this hyperthermophilic bacterium (Marszalkowski *et al.*, 2008). The universality of RNase P is also challenged in the archaeon *Nanoarchaeum equitans* in which tRNAs are transcribed as primary 5′ mature tRNAs, and therefore, RNase P activity has been dispensed (Randau *et al.*, 2008). In eukaryotes, a different exception occurs. Human mitochondria and higher plant chloroplasts possess a protein-only version of the enzyme, known as 'Proteinaceous RNase P', which lacks the RNA subunit (Holzmann *et al.*, 2008; Gobert *et al.*, 2010). In this case, RNase P enzymes seem to have lost the RNA component during evolution.

Despite less efficiently than with tRNAs, RNase P has been shown to cleave other substrates, both *in vivo* and *in vitro*. Namely, the *E. coli* enzyme processes two other important stable RNA substrates involved in protein synthesis: the tmRNA (Gimple & Schon, 2001) and 4.5S RNA (Bothwell

et al., 1976; Peck-Miller & Altman, 1991). Other substrates include phage-induced regulatory RNAs (Hartmann *et al.*, 1995), sRNA duplex substrates and snoRNAs (Ko & Altman, 2007; Yang & Altman, 2007), riboswitches (Altman *et al.*, 2005; Seif & Altman, 2008) and intergenic regions of polycistronic operon mRNAs (Alifano *et al.*, 1994; Li & Altman, 2003).

Catalysis by RNase P RNA is hydrolytic and absolutely dependent on divalent metal ions (Mg^{2+} or Mn^{2+}) (Smith *et al.*, 1992; Kirsebom & Trobro, 2009). Its turnover rate is slow compared with other enzymes, what may reflect a specialization for cleavage-site selectivity and recognition of several different substrates rather than for rapid catalysis. This would explain the complex nature of this ancient ribozyme.

RNase Z

RNase Z is a conserved endonuclease that belongs to the β-lactamase superfamily of metal-dependent hydrolases (Fig. 1a). Genes encoding RNase Z homologues were identified in all three domains of life (Minagawa *et al.*, 2004; de la Sierra-Gallay *et al.*, 2005). The enzyme is mainly responsible for the 3′ end maturation of tRNAs.

Mature tRNAs all bear a CCA sequence at the end of the acceptor stem that is essential for aminoacylation and interaction with the ribosome. Two main modes for 3′ tRNA processing have been described: (1) a one-step maturation involving direct endonucleolytic cleavage by RNase Z at the 3′ end (CCA less tRNAs). The cleavage occurs after the discriminator base (the unpaired nucleotide immediately upstream the CCA motif) (Nashimoto, 1997; Pellegrini *et al.*, 2003) and provides the substrate for subsequent CCA addition by tRNA nucleotidyltransferase to generate the mature tRNA (Deutscher, 1990; Nashimoto, 1997; Schiffer *et al.*, 2002); and (2) multistep maturation involving endo- and exonucleases (e.g. in *E. coli* where all genes have the CCA encoded). Hence, the presence or not of the universal 3′-terminal CCA sequence in the tRNA primary transcript is the key determinant for the 3′-tRNA processing pathway (Deutscher, 1990; Schiffer *et al.*, 2002). In organisms such as *B. subtilis*, both types of 3′-tRNA processing may occur (see the section below on processing).

While the RNase Z gene is essential in *B. subtilis* for cell viability (Schilling *et al.*, 2004), in *E. coli*, mutants lacking RNase Z have no obvious growth phenotype (Schilling *et al.*, 2004). The *E. coli* RNase Z, also known as the ElaC protein, was initially identified as a zinc phosphodiesterase, ZiPD (Vogel *et al.*, 2002; Schilling *et al.*, 2004). It had been identified several years before as RNase BN, initially thought to be a cobalt-activated RNase with exonuclease activity (Asha *et al.*, 1983). The enzyme was required for the maturation of tRNA precursors encoded by phage T4. However, the gene encoding RNase BN (*rbn*) was originally misidentified, and was only recently shown to be the *elaC* gene, known to encode RNase Z (Ezraty *et al.*, 2005). Therefore, the *E. coli* enzyme is still called RNase BN occasionally. Other denominations include tRNase Z, 3′-tRNase and 3′-pre-tRNase.

The enzyme is a zinc-dependent metallo-hydrolase, and like RNase P, recognizes the tRNA structure in precursor molecules (Pellegrini *et al.*, 2003). RNase Z crystal structures have revealed that the enzyme forms a dimer of metallo-β-lactamase domains and has a characteristic domain, named a flexible arm or an exosite, which protrudes from the metallo-β-lactamase core and is involved in tRNA binding (de la Sierra-Gallay *et al.*, 2005). In the case of *Thermotoga maritima*, the structure of the flexible arm of the enzyme is different from those of homologue enzymes and may explain why, in this bacterium, tRNase Z exceptionally cleaves precisely after the CCA sequence (at 3′) and not after the discriminator base (Ishii *et al.*, 2005).

The intriguing presence of an RNase Z homologue in some members of the *Gammaproteobacteria*, such as *E. coli* and *Salmonella* spp., even though its action is not needed for tRNA maturation, has led to a search for other potential substrates for RNase Z. Surprising results were obtained when the *rnz* mutation was combined with a mutation in RNase E. The lack of both enzymes resulted in a drastic increase in the half-live compared with the absence of either enzyme alone (Perwez & Kushner, 2006a). These authors also observed that *E. coli* RNase Z was able to cleave *rpsT* mRNA *in vitro* at locations distinct from those obtained with RNase E. The enzyme is also capable of cleaving unstructured RNA substrates (Shibata *et al.*, 2006).

Deutscher and coworkers proposed that the *E. coli* enzyme (RNase BN) may differ in certain respects from the RNase Z homologues in other organisms; namely, it can have a dual exo- and an endoribonuclease activity (Dutta & Deutscher, 2009, 2010). This dual activity was also seen in RNase J from *B. subtilis*, another member of the zinc-dependent metallo-β-lactamases family (see the section on Other endonucleases) (Mathy *et al.*, 2007).

Other endonucleases

Several other endonucleases have been described not only in *E. coli* but also in other microorganisms. Below, we will briefly mention some of their main characteristics.

RNase I is a broad-specificity endoribonuclease, very active, present in the periplasmic space of *E. coli*. The enzyme belongs to the T2 superfamily of RNases, whose members are widely distributed throughout nature (Irie, 1997; Condon & Putzer, 2002) (Fig. 1a). Although RNase I activity is easily detected, its function in cell metabolism has never been clarified, because RNase I-deficient mutants are viable and do not affect global mRNA degradation (Zhu

et al., 1990). The enzyme can cleave RNA between every residue to yield mononucleotides and its activity is not inhibited in the presence of EDTA. It was proposed to be implicated in the scavenging of ribonucleotides from the extracellular environment (Condon & Putzer, 2002).

There are reports of other broad-specificity endoribonucleases that are RNase I related, namely, RNase I* (Cannistraro & Kennell, 1991) and RNase M (Cannistraro & Kennell, 1989). However, their existence was never confirmed and seems to consist merely of different manifestations of RNase I (Subbarayan & Deutscher, 2001).

Escherichia coli RNase LS is an RNase that, despite playing a minor role in noninfected bacteria (reviewed in Uzan, 2009), seems to constitute an important cellular defense mechanism against bacteriophage invasion (Otsuka & Yonesaki, 2005). Namely, bacteriophage T4 uses a combination of host- and phage-encoded enzymes to degrade its mRNAs in a stage-dependent manner. Phage T4 encodes RegB, a sequence-specific endoribonuclease (Sanson & Uzan, 1995; Uzan, 2001) that inactivates T4 early transcripts shortly after infection. The middle and late T4 mRNAs are protected from degradation by the viral factor Dmd. In T4-phages defective for the *dmd* gene, RNase LS (for late-gene silencing in T4) cleaves these T4 mRNAs, inhibiting phage multiplication. Therefore, this endonuclease acts as an antagonist of T4 phage replication and Dmd is required for overcoming the host's RNase LS defense role.

Escherichia coli also encodes for a large number of suicide or toxin genes. Their expression is toxic to their host cells, causing growth arrest and eventual cell death. For example, *E. coli* RelE and MazF are two different families of bacterial toxins that inhibit translation by specific endonucleolytic mRNA cleavage (Pedersen *et al*., 2003; Neubauer *et al*., 2009; Yamaguchi & Inouye, 2009).

In *B. subtilis*, it was shown that the majority of the ribonucleolytic activity is phosphorolytic. However, several studies showed that PNPase is not responsible for the initial step in RNA decay in *B. subtilis*, but is a secondary enzyme that acts after the decay has been initiated by other RNases (Bechhofer, 2009). Recently, two proteins (RNase J1 and RNase J2) with cleavage activity equivalent to *E. coli* RNase E were purified in this organism (Even *et al*., 2005). Moreover, these enzymes share many other characteristics with RNase E, which may be related to their similar endonucleolytic activities (Bechhofer, 2009). RNase J1 and J2 are around 61 kDa and have both endonucleolytic and 5′–3′ exonucleolytic activity, which is sensitive to the 5′ phosphorylation state of the substrate. These enzymes were the first described 5′–3′ exonucleases in bacteria (Mathy *et al*., 2007), the J1 activity being twofold higher than J2 (Mathy *et al*., 2010) (see also under the topic Exonucleases the section on RNase J1/J2). Furthermore, RNase J1 is essential, while RNase J2 is not (Even *et al*., 2005).

RNase J1 plays a major role in RNA stability (Mader *et al*., 2008) and maturation. It functions as a 5′–3′ exoribonuclease in the maturation of 16S rRNA gene and in regulating the mRNA stability of the stationary-phase insecticidal protein transcript *cryIIIA* (Mathy *et al*., 2007; Deikus *et al*., 2008). RNase J1 is also responsible for increasing the stability of the downstream fragments that result from the endonucleolytic cleavage of *thrS* and *thrZ* mRNAs (Even *et al*., 2005). A recent study using a bacterial two-hybrid system showed that PNPase, RNase J1 and two glycolytic enzymes can interact with RNase Y and potentially form a degradosome-like complex (Commichau *et al*., 2009) (see Complexes of RNases). Moreover, it was shown recently that RNase J1 and J2 in wild-type cells are mostly in a complex. While the individual enzymes have similar endonucleolytic cleavage activities and specificities, as a complex, they behave synergistically to alter cleavage site preference and to increase cleavage efficiency at specific sites (Mathy *et al*., 2010).

RNase J1 homologues are widely distributed in several other bacteria and archaea (Even *et al*., 2005). The enzyme is a member of the β-CASP subfamily of zinc-dependent metallo-β-lactamases. The enzyme is composed of three domains: an N-terminal β-lactamase domain, a β-CASP and a C-terminal domain necessary for the enzyme activity. A binding pocket coordinating the phosphate and base moieties of the nucleotide in the surrounding area of the catalytic center provides a basis for the 5′-monophosphate-dependent 5′–3′ exoribonuclease activity (de la Sierra-Gallay *et al*., 2008). The endonucleolytic activity of the enzyme is not dependent of 5′-monophosphate. For the initiation of endonuclease cleavage, RNase J1 either binds to the 5′ end or directly to the internal site of the mRNA. The upstream product is rapidly degraded by the 3′–5′ exonuclease activity of PNPase. The downstream RNA fragment with the 5′-monophosphate end can be a target of new RNase J1 endonuclease cleavage or processive 5′–3′ exonucleolytic decay from the 5′ end (Bechhofer, 2009). It was also shown that RNase J1 requires a single-stranded 5′ end with AU-rich regions to allow the exoribonucleolytic activity (Mathy *et al*., 2007). This was observed in *infC* leader RNA (Choonee *et al*., 2007), *trp* leader RNA (Deikus *et al*., 2008) and the RNA species called scRNA (Yao *et al*., 2007).

Similar to what happens with *B. subtilis*, we can find RNase J1 and J2 also in *Streptococcus pyogenes*. While in *B. subtilis* only RNase J1 is an essential protein, in *S. pyogenes*, both proteins are essential for growth. In this bacterium, RNases J1 and J2 were also seen to affect the decay of several mRNAs (Bugrysheva & Scott, 2009).

Another endonuclease sensitive to the 5′ end phosphorylation state of the substrate was discovered recently. RNase Y is involved in the initiation of turnover of *B. subtilis S*-adenosylmethionine-dependent riboswitches (Shahbabian

et al., 2009), which controls the expression of 11 transcriptional units (Winkler & Breaker, 2005; Henkin, 2008). The enzyme has a major function in the initiation of mRNA degradation in this organism, affecting mRNA stability > 30% in an RNase J1/J2 double-mutant strain. RNase Y orthologues are present in about 40% of the eubacteria; however, this enzyme is absent from archaea and eukaryotic organisms, with the exception of *Drosophila willistoni* (Shahbabian *et al.*, 2009).

Other endonucleases are described in *B. subtilis* such as RNase M5, RNase Z (see the above section on RNase Z), RNase Bsn and RNase P (see the above section on RNase P). However, neither RNase M5 nor RNase Z appears to have mRNA targets in *B. subtilis* (Condon *et al.*, 2002). RNase M5's major role is the maturation of the 5S rRNA gene (Sogin & Pace, 1974) and can only be found in low G+C Gram-positive bacteria (Condon *et al.*, 2001). Bsn is an extracellular nuclease, apparently with no sequence specificity. It can cleave RNA endonucleolytically to yield 5′-phosphorylated oligonucleotides. The enzyme is found in some members of low G+C Gram-positive bacteria (Nakamura *et al.*, 1992).

Barnase is a guanyl-specific extracellular RNase. Although it is found in many of the *Bacilli*, it is not present in *B. subtilis*. Orthologues of *Bacillus amyloliquefaciens* Barnase and its inhibitor Barstar are also found in *Clostridium acetobutylicum* and the Gram-positive *Yersinia pestis*. It appears that some organisms have lost their copy of the Barnase gene because it was no longer required for a selective advantage. Alternatively, they acquired the resistance gene because other organisms sharing the same niche produced Barnase (Belitsky *et al.*, 1997).

Besides the well-known endonucleases, there are some DNA-binding proteins in archaea with RNase endonucleolytic activity; however, the physiological relevance of these proteins with respect to RNA metabolism is not clear (Evguenieva-Hackenberg & Klug, 2009). The attempts to purify novel RNase activities from archaea resulted in the isolation of very different proteins. Two proteins with RNase activity were purified from *Sulfolobus solfataricus* (called p1 and p2). It was shown that divalent cations are not required for their activity, and they were capable of cleaving yeast tRNA (Fusi *et al.*, 1993; Shehi *et al.*, 2001). Another 9-kDa protein, called SaRD, whose RNase activity is not affected in the presence of different divalent cations, was purified from *Sulfolobus acidocaldarius* (Kulms *et al.*, 1995). Furthermore, two different dehydrogenases were identified in the same organism, with RNase III-like properties and cleavage patterns dependent on $MgCl_2$: an aspartate-semialdehyde dehydrogenase and acyl-CoA dehydrogenase (Evguenieva-Hackenberg *et al.*, 2002). A homologue of the eukaryotic initiation factor 5A (eIF-5A) called archaeal initiation factor 5A (aIF-5A), from *Halobacterium salinarum*, was also described as an RNase with activity in low salt concentrations without addition of $MgCl_2$ (Wagner & Klug, 2007). It was shown that aIF5A efficiently binds structured RNA containing certain motifs and that the interaction is hypusine dependent (Xu *et al.*, 2004).

Exonucleases

PNPase

PNPase belongs to the PDX family of exoribonucleases, which also includes RNase PH from bacteria, and the core of the exosome in archaea and eukaryotes (Mian, 1997; Zuo & Deutscher, 2001; Pruijn, 2005) (Fig. 1b). In 1959, Severo Ochoa received the Nobel Prize for his studies on the polymerase activity of this enzyme, being the first to synthesize RNA outside the cell. This was a major contribution towards deciphering the genetic code. PNPase is also involved in global mRNA decay, being widely conserved from bacteria to plants and metazoans (Zuo & Deutscher, 2001; Bermúdez-Cruz *et al.*, 2005).

PNPase is encoded by the *pnp* gene and is transcribed from two promoters (Portier & Regnier, 1984). *pnp* expression is negatively autoregulated at the post-transcriptional level by the concerted action of PNPase and RNase III (Portier *et al.*, 1987; Robert-Le Meur & Portier, 1992, 1994; Jarrige *et al.*, 2001; Carzaniga *et al.*, 2009). This autoregulation can be disrupted by ribosomal protein S1, which binds to the *pnp* mRNA 5′-UTR (Briani *et al.*, 2008). In an RNase III-deficient strain, there is a 10-fold increase in the PNPase levels (Portier *et al.*, 1987). PNPase levels are also affected by polyadenylation. It is likely that polyadenylated transcripts titrate out the amount of PNPase available to carry out normal autoregulation (Mohanty & Kushner, 2002). PNPase and RNase II are cross-regulated (Zilhão *et al.*, 1996a). In the absence of RNase II, PNPase levels are increased and PNPase overexpression leads to a decrease in RNase II activity (Zilhão *et al.*, 1996a).

PNPase does not seem to be indispensable to *E. coli* at optimal temperature, unless either RNase II or RNase R is also missing (Donovan & Kushner, 1986; Cheng *et al.*, 1998). However, PNPase is essential for *E. coli* growth at low temperatures (Luttinger *et al.*, 1996; Piazza *et al.*, 1996; Zangrossi *et al.*, 2000) and certain mutations of the RNA-binding domains have been shown to confer a cold-sensitive phenotype (García-Mena *et al.*, 1999; Briani *et al.*, 2007; Matus-Ortega *et al.*, 2007). Higher levels of RNase II allow lower levels of PNPase, and in fact, overexpression of RNase II could complement the cold-shock function of PNPase (Zilhão *et al.*, 1996a; Awano *et al.*, 2008). PNPase was also shown to be involved in the long-term survival of *Campylobacter jejuni* at temperatures > 10 °C (Haddad *et al.*, 2009). In *E. coli*, cold-temperature induction of *pnp* expression occurs at post-transcriptional levels including the reversal of

pnp autoregulation (Zangrossi *et al.*, 2000; Beran & Simons, 2001; Mathy *et al.*, 2001).

PNPase processively catalyzes the 3′–5′ phosphorolytic degradation of RNA, releasing nucleoside 5′-diphosphates. Although the degrading activity of *E. coli* PNPase is known to be blocked by dsRNA structures (Spickler & Mackie, 2000), PNPase can form complexes with other proteins, allowing it to degrade through extensive structured RNA. The main multiprotein complex that integrates PNPase is the degradosome (see the Complexes of RNases). To degrade certain dsRNAs, PNPase can form a complex ($\alpha_3\beta_2$) with RNA helicase B (RhlB) (Liou *et al.*, 2002; Lin & Lin-Chao, 2005). PNPase also forms complexes with Hfq and PAP I (Mohanty *et al.*, 2004). The enzyme was reported to degrade a stem-loop without the assistance of RhlB, but this could be related to the low thermodynamic stability of the stem-loop (Mohanty & Kushner, 2010). In the Gram-negative bacteria *Thermus thermophilus*, the PNPase homologue (Tth PNPase) was shown to have phosphorolytic activity at the optimal temperature of 65 °C. Surprisingly, it is able to completely degrade RNAs with very stable intramolecular secondary structures (Falaleeva *et al.*, 2008).

A minimal 3′ overhang of 7–10 unpaired ribonucleotides is required for an RNA molecule to be bound by PNPase (Py *et al.*, 1996; Cheng & Deutscher, 2005), and the action of the enzyme on folded RNAs is known to be stimulated by 3′ polyadenylation (Xu & Cohen, 1995; Py *et al.*, 1996; Carpousis *et al.*, 1999; Spickler & Mackie, 2000). PNPase is also able to catalyze the polymerization of RNA from nucleoside diphosphates at a low inorganic phosphate (Pi) concentration (Godefroy, 1970; Littauer & Soreq, 1982; Sulewski *et al.*, 1989). *In vivo*, PNPase is essentially devoted to the processive degradation of RNA, but is also responsible for adding the heteropolymeric tails observed in *E. coli* mutants devoid of the main polyadenylating enzyme PAP I (Mohanty & Kushner, 2000b; Slomovic *et al.*, 2008). In exponentially growing *E. coli*, $>$ 90% of the transcripts are polyadenylated and Rho-dependent transcription terminators were suggested to be modified by the polymerase activity of PNPase (Mohanty & Kushner, 2006). In spinach chloroplasts, *Cyanobacteria* and *Streptomyces coelicolor*, PNPase seems to be the main tail polymerizing enzyme (Yehudai-Resheff *et al.*, 2001; Rott *et al.*, 2003; Sohlberg *et al.*, 2003). PNPase-dependent RNA tailing and degradation are believed to occur mainly at low ATP concentrations, because ATP has been shown to inhibit both activities (Del Favero *et al.*, 2008). Recently, it was shown that *B. subtilis* PNPase, in the presence of Mn^{2+} and low levels of Pi, is also able to degrade ssDNA, while in the presence of Mg^{2+} and higher amounts of Pi, it degrades RNA. This suggests that PNPase degradation of RNA and ssDNA occurs by mutually exclusive mechanisms (Cardenas *et al.*, 2009). Because of the ability of PNPase to carry out several distinct activities, the enzyme can be considered as a multifunctional protein. It is a pleiotropic regulator, involved in a number of different pathways of RNA degradation. Indeed, it is the only exoribonuclease in *Streptomyces* and is an essential enzyme in these organisms (Bralley & Jones, 2003; Bralley *et al.*, 2006). In *E. coli*, PNPase is now believed to play a greater role in mRNA degradation than previously thought and its inactivation increases the steady-state levels of many transcripts (Deutscher & Reuven, 1991; Mohanty & Kushner, 2003). The enzyme was also reported to play an important role in protecting *E. coli* cells under oxidative stress (Wu *et al.*, 2009). In *B. subtilis*, the RNA decay is primarily phosphorolytic and this major activity is attributed to the PNPase, which is the principal 3′–5′ exoribonuclease in this organism. The deletion of PNPase in *B. subtilis* causes a number of phenotypes such as competence deficiency, cold and tetracycline sensitivity, and filamentous growth (Hahn *et al.*, 1996; Luttinger *et al.*, 1996; Wang & Bechhofer, 1996).

X-ray crystal structures of *E. coli* and *Streptomyces antibioticus* PNPase reveal a homotrimeric subunit organization with a ring-like architecture (Symmons *et al.*, 2000; Shi *et al.*, 2008; Nurmohamed *et al.*, 2009). Each monomer exhibits a five-domain arrangement: at the N-terminus, two RNase PH domains (PH1 and PH2) are linked by an α-helical domain; two RNA-binding domains, KH and S1, are found in the C-terminal end. In the quaternary structure, the KH and S1 domains are found together in one face of the trimer, while the active site is found in the opposite side.

PNPase mutants lacking either the S1 or the KH domain retain phosphorolytic activity (Jarrige *et al.*, 2002; Stickney *et al.*, 2005; Matus-Ortega *et al.*, 2007). However, the presence of both KH and S1 domains is required for a proper binding (Matus-Ortega *et al.*, 2007), and their absence was proposed to affect product release and enzyme cycling, leading to a decreased turnover number (Stickney *et al.*, 2005). The crystal structure of a KH/S1 deletion mutant, along with biochemical and biophysical data, strongly suggests that these domains are involved not only in RNA binding but also contribute to the formation of a more stable trimeric structure (Shi *et al.*, 2008). Indeed, a previous study has shown that the S1 domain from PNPase was able to induce trimerization of a chimeric RNase II containing PNPase S1 (Amblar *et al.*, 2007).

The association of the three subunits encloses a central channel. A properly constricted channel and the conserved basic residues located in the neck region have been shown to play critical roles in trapping RNA for processive degradation (Shi *et al.*, 2008). Two constricted points have been identified in the channel, and the structure of PNPase in complex with RNA clearly indicates that the pathway followed by the RNA molecule is along the central pore in the direction of the active site (Symmons *et al.*, 2000; Shi *et al.*, 2008; Nurmohamed *et al.*, 2009). The ability of the

aperture at the central channel and its neighboring regions to undergo conformational changes is likely to be a key aspect of the dynamic translocation of RNA by PNPase (Nurmohamed *et al.*, 2009).

The catalytic site of PNPase is composed of structural elements of both PH1 and PH2 core domains, and several mutations introduced into the PNPase core abolish or drastically decrease all catalytic activities of the enzyme (Jarrige *et al.*, 2002; Briani *et al.*, 2007). However, other mutations in the core region were analyzed that do not affect phosphorolytic or polymerase activities, but rather RNA binding is severely impaired (Regonesi *et al.*, 2004). *Streptomyces antibioticus* PNPase catalytic center has been identified using tungstate (a phosphate analogue), which is coordinated by T462 and S463 (Symmons *et al.*, 2000). *Escherichia coli* PNPase crystals obtained in the presence of Mn^{2+} (which can substitute for Mg^{2+} to support catalysis) showed that the metal is coordinated by the conserved residues D486, D492 and K494 (Nurmohamed *et al.*, 2009). Indeed, the substitution of D492 abolished both phosphorolysis and polymerization activities (Jarrige *et al.*, 2002).

PNPase has been described to play a role in the establishment of virulence in several pathogens. In *Salmonella*, PNPase activity decreases the expression of genes from the pathogenicity islands SPI 1 (containing genes for invasion) and SPI 2 (containing genes for intracellular growth) (Clements *et al.*, 2002). Similarly, in *Dichelobacter nodosus*, PNPase acts as a virulence repressor in benign strains by decreasing twitching motility (Palanisamy *et al.*, 2009). In contrast, in *Yersinia*, PNPase modulates the type three secretion system (TTSS) by affecting the steady-state levels of TTSS transcripts and controlling the secretion rate (Rosenzweig *et al.*, 2005, 2007). This is probably the reason why the *pnp* deletion results in a less virulent strain in a mouse model (Rosenzweig *et al.*, 2007). In *C. jejuni* PNPase is involved in motility (Haddad *et al.*, 2009). Finally, in *S. pyogenes*, PNPase activity is rate-limiting for the decay of *sagA* and *sda*, which code for the important virulence factors streptolysin S and streptodornase (a DNase), respectively (Barnett *et al.*, 2007).

RNase II

Escherichia coli RNase II is the prototype of the RNase II family of enzymes (Mian, 1997; Mitchell *et al.*, 1997; Zuo & Deutscher, 2001; Frazão *et al.*, 2006; Grossman & van Hoof, 2006) (Fig. 1b). RNase II-like proteins are widespread among the three domains of life, and in eukaryotes, they are the catalytic component of the exosome (Liu *et al.*, 2006b; Dziembowski *et al.*, 2007).

RNase II is encoded by the *rnb* gene that can be transcribed from two promoters P1 and P2 and terminates in a Rho-independent terminator 10 nucleotides downstream of the *rnb* stop codon (Zilhão *et al.*, 1993, 1995a, 1996b). PNPase regulates RNase II expression by degrading the *rnb* mRNA (Zilhão *et al.*, 1996a). RNase III and RNase E endonucleases are also involved in the control of RNase II expression at the post-transcriptional level. RNase III does not affect *rnb* mRNA directly, but affects PNPase levels, and RNase E is directly involved in the *rnb* mRNA degradation (Zilhão *et al.*, 1995b).

The protein stability of RNase II is known to be post-translationally regulated and its levels are adjusted according to the growth conditions. *gmr* (gene modulating RNase II) is located downstream of *rnb* and the related protein is involved in the modulation of the stability of RNase II (Cairrão *et al.*, 2001). Gmr has a PAS domain that can act as an environmental sensor detecting changes in growth conditions.

Escherichia coli RNase II is a sequence-independent hydrolytic exoribonuclease that processively degrades RNA in the 3′–5′ direction, yielding 5′-nucleoside monophosphates. However, the processive degradation of an RNA molecule by RNase II is easily blocked by secondary structures, and the enzyme is known to stall around seven nucleotides before it reaches a double-stranded region (Cannistraro & Kennell, 1999; Spickler & Mackie, 2000). In *E. coli*, RNase II is the major hydrolytic enzyme and participates in the terminal stages of mRNA degradation (Deutscher & Reuven, 1991). However, the enzyme is not essential for *E. coli* growth unless PNPase is also missing (Donovan & Kushner, 1986; Zilhão *et al.*, 1996a). Although RNase II-degrading activity is sequence independent, the most reactive substrate is the homopolymer poly(A). Because the presence of a poly(A) tail is often needed for the RNA degradative process, the rapid degradation of polyadenylated stretches by RNase II can paradoxically protect some RNAs by impairing the access of other exoribonucleases (Hajnsdorf *et al.*, 1994; Pepe *et al.*, 1994; Coburn & Mackie, 1996a; Marujo *et al.*, 2000; Mohanty & Kushner, 2000a; Folichon *et al.*, 2005). Indeed, in the absence of RNase II, a large number (31%) of *E. coli* mRNAs are decreased, especially ribosomal protein genes, suggesting a major function for this enzyme in the protection of specific mRNAs through poly(A) tail removal (Mohanty & Kushner, 2003).

The structure of *E. coli* RNase II and its RNA-bound complex was determined (Frazão *et al.*, 2006) (Fig. 2a). This was the first structure of an exoribonuclease from the RNase II family that has been resolved (Frazão *et al.*, 2006). The overall X-ray crystallographic structure of the wild-type enzyme (Frazão *et al.*, 2006; Zuo *et al.*, 2006) revealed four domains, as predicted previously by Amblar *et al.* (2006) (see Figs 1b and 2a). Three RNA-binding domains have been identified: two cold-shock domains (CSD1 and CSD2) in the N-terminal region and an S1 RNA-binding domain at

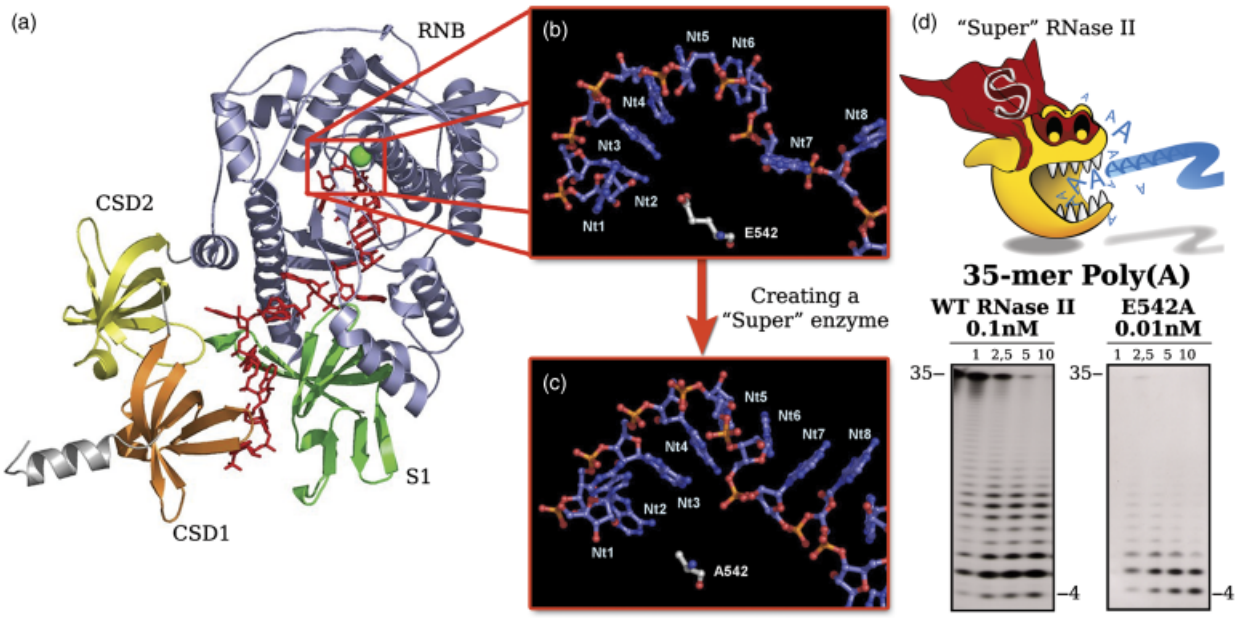

Fig. 2. The making of a 'super-enzyme'. (a) RNase II is composed of two N-terminal cold shock domains (CSD1 in orange and CSD2 in yellow), a central catalytic domain (RNB in gray), a C-terminal S1 domain (in green). (b) Zooming the catalytic cavity of RNase II. (c) Modelling the E542A mutant with the Poly(A) RNA strand in the RNB domain. Substitution in position 542 of the negatively charged glutamic side-chain for the smaller neutral methyl group of alanine could reduce significantly both electrostatic and steric surfaces in the RNA-binding interface. (d) Exoribonuclease activity with the Poly(A) substrate: comparison of wild-type and E542A proteins. It is possible to see that we need to use higher concentrations of RNase II when compared with the E542A mutant, which is 110-fold more active when compared with the wild type (Barbas *et al.*, 2009. ©The American Society for Biochemistry and Molecular Biology).

the C-terminus. The catalytic site resides in the central RNB domain, whose structure has shown an unprecedented fold characteristic of this family. This domain contains four highly conserved sequence motifs (I–IV) with several invariant carboxylate residues (Mian, 1997). The RNA-binding domains (CSD1, CSD2 and S1) are grouped together on one side of the structure, while the active site is on the other side of the molecule (Frazão *et al.*, 2006).

Elimination of the N-terminal CSD1 resulted in an increase in the RNA-binding affinity of the enzyme for poly(A), suggesting that this domain may play a role in controlling the movement of the enzyme on the poly(A) chain (Amblar *et al.*, 2006; Arraiano *et al.*, 2008). Interestingly, without all the RNA-binding domains, the enzyme is still able to degrade RNA, although with much less efficiency than the wild-type enzyme (Matos *et al.*, 2009; Vincent & Deutscher, 2009).

The structure of the RNA-bound enzyme revealed that the RNA fragment interacts with the protein at two non-contiguous regions: the 'anchor' and catalytic regions (Cannistraro & Kennell, 1994; Frazão *et al.*, 2006) (Fig. 2a). Nucleotides 1–5, at the 5′ end of the 13-mer RNA fragment, are located in the 'anchor' region in a deep cleft between the two CSDs and the S1 domain. The final nucleotides 9–13 are located in a cavity deep within the RNB domain, stacked and 'clamped' between the conserved residues Phe358 and Tyr253. A 10-nucleotide fragment is the shortest RNA able to retain contacts with both the anchor and the catalytic regions. This explains why RNase II is processive on long RNA molecules, but becomes distributive on substrates shorter than 10–15 nucleotides. When the RNA molecule is shorter than five nucleotides, the required packing of the bases can no longer occur, preventing the translocation of the RNA, and a final end product of four nucleotides is released (Frazão *et al.*, 2006). Tyr-253 has been identified as the residue responsible for setting the RNase II end product, and its substitution was shown to alter the smallest end product of degradation from 4 to 10 nucleotides (Barbas *et al.*, 2008). This mutation has been proposed to cause loosening of the RNA substrate at the catalytic site and, as a consequence, binding at the anchor region would be essential to keep the RNA attached to the protein and allow cleavage. Molecules shorter than 10 nucleotides are too small to be simultaneously bound at both sites, meaning that they would have to be degraded in a distributive manner (Barbas *et al.*, 2008).

The access to the catalytic pocket is restricted to single-stranded RNA by steric hindrance, which explains the inability of RNase II to degrade dsRNA. DNA is not a substrate because there is a specific interaction between the protein and the ribose rings of nucleotides that directly contact the enzyme (Frazão *et al.*, 2006). Residues Tyr-313

and Glu-390 have been demonstrated to be responsible for the discrimination of the cleavage of RNA vs. DNA (Barbas *et al.*, 2009).

Several residues in the catalytic region are important for catalysis (Amblar & Arraiano, 2005; Frazão *et al.*, 2006). Asp-201 and Asp-210 substitution led to a significant loss of RNase II activity, and Arg-500 has also been shown to be crucial for RNA cleavage (Frazão *et al.*, 2006; Barbas *et al.*, 2008, 2009). However, Asp-209 is the only essential residue for RNA degradation (Barbas *et al.*, 2008). The conserved residue Glu-542 has been proposed to facilitate the elimination of the exiting nucleotide upon phosphodiester cleavage (Frazão *et al.*, 2006). Interestingly, its substitution by alanine rendered the mutant RNase II much more active than the wild type and significantly increased the RNA-binding ability (Fig. 2b–d). Three-dimensional modelling of the mutant enzyme indicated that the substitution induced a subtle conformational change in the RNB domain. This resulted in a reorganization of the RNA-binding interface that transformed the RNase II into the so-called 'super-enzyme', an enzyme with extraordinary catalysis and binding abilities. When compared with the wild-type RNase II, the 'super-enzyme' exhibits >100-fold increase in the exoribonucleolytic activity (Fig. 2d) and about a 20-fold increase in the RNA-binding affinity (Barbas *et al.*, 2009).

RNase R

RNase R encoded by the *rnr* gene (previously *vacB*) is a 3′–5′ hydrolytic exoribonuclease from the RNase II family of exoribonucleases (Cheng & Deutscher, 2002; Vincent & Deutscher, 2006). The *rnr* gene is second in an operon, together with *nsrR* (a transcriptional regulator), *rlm*B (rRNA methyltransferase) and *yjfI* (unknown function). Transcription is driven from a putative σ^{70} promoter upstream of *nsrR* (Cheng *et al.*, 1998; Cairrão *et al.*, 2003). *rnr* mRNAs are post-transcriptionally regulated by RNase E, although RNase G may also participate (Cairrão & Arraiano, 2006). RNase R is a processive and sequence-independent enzyme, with a wide impact on RNA metabolism (Cairrão *et al.*, 2003; Cheng & Deutscher, 2005; Oussenko *et al.*, 2005; Andrade *et al.*, 2006, 2009a; Purusharth *et al.*, 2007). It is unique among the RNA-degradative exonucleases present in *E. coli* as it can easily degrade highly structured RNAs (Cheng & Deutscher, 2002, 2003; Awano *et al.*, 2010). RNase R is able to degrade an RNA duplex, provided there is a single-stranded 3′ overhang (Cheng & Deutscher, 2002; Vincent & Deutscher, 2006). In fact, RNase R was shown to be a key enzyme involved in the degradation of polyadenylated RNA (Andrade *et al.*, 2009a).

RNase R shows a modular organization of RNA-binding domains (CSD1 and CSD2 located at the N-terminus and a C-terminal S1 domain) flanking the central catalytic RNB domain, typically found on RNase II-family members (Fig. 1b). A three-dimensional model of RNase R has been proposed based on the structure of its paralogue RNase II (Barbas *et al.*, 2008). Mutational analysis identified important residues located in the active center: D272, D278 and D280 (Matos *et al.*, 2009). A D280N mutant showed no exonucleolytic activity, similarly to what was reported with the D209N mutant in RNase II (Amblar & Arraiano, 2005; Matos *et al.*, 2009; Awano *et al.*, 2010). RNase R degradation is processive, and unlike RNase II, the final end product of digestion is a dinucleotide. Tyrosine Y324 was found to be responsible for setting the final end product of RNase R (Matos *et al.*, 2009).

RNase R was shown to bind RNA more tightly within its catalytic channel than does RNAase II (Matos *et al.*, 2009; Vincent & Deutscher, 2009). Surprisingly, a mutant expressing only the nuclease domain (RNB) is able to degrade a perfect dsRNA (Matos *et al.*, 2009). Paradoxically, the presence of the RNA-binding domains (CDS1, CDS2 and S1) requires the presence of a short tail in order to degrade dsRNA (Matos *et al.*, 2009). The RNA-binding domains 'block' the entrance of dsRNA into the catalytic channel. Accordingly, it was proposed that RNA-binding domains actually discriminate the substrates that can be processed by RNase R, favoring the selection of RNA molecules harboring a 3′ linear tail. It has been suggested that RNase R can function both as an exoribonuclease as well as an RNA 'helicase' (Awano *et al.*, 2010). RNase R intrinsic 'helicase' unwinding activity is dependent on RNA-binding regions (S1, CDS1, and most importantly, CDS2). The dsRNA must have a 3′ linear overhang in order to become a suitable substrate for RNase R helicase activity. Altogether, RNA-binding domains of RNase R seem to be responsible for the selection of RNA substrates harboring a 3′ linear region, which can be provided by polyadenylation (Andrade *et al.*, 2009a; Matos *et al.*, 2009). Clearly, only the resolution of the RNase R structure will allow a full understanding of its remarkable modes of action.

RNase R is critical in RNA quality control, namely in the degradation of defective tRNAs (Vincent & Deutscher, 2006; Awano *et al.*, 2010) and rRNA (Cheng & Deutscher, 2003). Together with PNPase, RNase R eliminates aberrant fragments of the 16S and 23S rRNA genes, whose accumulation potentially affects ribosome maturation and assembly. Furthermore, the importance of RNase R in the accuracy of gene expression is broadened with its role in protein quality control. In the absence of RNase R, the small stable SsrA/tmRNA is not processed properly, leading to defects in *trans*-translation and significant errors in protein tagging for proteolysis (Cairrão *et al.*, 2003). RNase R has also emerged as an important novel contributor to mRNA degradation. The absence of both RNase R and PNPase results in the strong accumulation of REP-containing mRNA sequences (Cheng & Deutscher, 2005). However, the presence of only

one of these exoribonucleases is sufficient to remove such transcripts, revealing again a functional overlap between these two enzymes. Remarkably, RNase R was also shown to degrade the *ompA* transcript in a growth-phase-specific manner (Andrade *et al.*, 2006). In the stationary phase of growth, the single inactivation of RNase R results in the accumulation of *ompA* mRNA and this correlated with increasing intracellular levels of OmpA protein. This work revealed a role for RNase R in the control of gene expression that could not be replaced by any of the other exoribonucleases (Andrade *et al.*, 2006).

The activity of RNase R is modulated according to the growth conditions of the cell and responds to environmental stimuli. RNase R seems to be a general stress-induced protein, whose levels are increased under several stresses, namely in cold shock, and the stationary phase of growth (Cairrão *et al.*, 2003; Andrade *et al.*, 2006). RNase R-like enzymes are widespread in most sequenced genomes. Although most of the knowledge on this protein came from work in *E. coli*, many RNase R from other bacterial species have been identified. Notably, RNase R has also been implicated in the establishment of virulence in a growing number of pathogens.

In *Shigella flexneri*, RNase R was shown to be required for the expression of the invasion factors IpaB, IpaC, IpaD and VirG (Tobe *et al.*, 1992). The disruption of the VacB gene in other *Shigella* spp. and enteroinvasive *E. coli* resulted in the reduced expression of virulence phenotypes (Tobe *et al.*, 1992). In *Legionella pneumophila* RNase R is the only hydrolytic exoribonuclease present. This protein is not essential for growth at optimal temperature; however, it is important for growth and viability at low temperatures and induces the competence (Charpentier *et al.*, 2008). To date, only one exoribonuclease, RNase R (MgR), was identified in *Mycoplasma genitalium*, where it is an essential protein (Hutchison *et al.*, 1999). MgR shares some properties of both *E. coli* RNase R and RNase II and can carry out a broad range of RNA processing and degradative functions (Lalonde *et al.*, 2007). Similar to what happens in *E. coli*, RNase R from *Aeromonas hydrophila* is also a cold-shock protein essential for viability at lower temperatures and its absence leads to a reduction in *A. hydrophila* motility (Erova *et al.*, 2008). The infection of mouse cells with Δ*rnr* strains shows that the virulence is attenuated, confirming the role of this enzyme in the pathogenesis of this organism (Erova *et al.*, 2008). In *Streptococcus pneumoniae*, there is a unique homologue of the RNase II family of enzymes that was shown to be a RNase R-like protein (Domingues *et al.*, 2009). RNase R from *Salmonella* showed a reduction in its activity and the ability to bind to RNA when compared with *E. coli* RNase R (Domingues *et al.*, 2009). Proteins isolated from different strains regarding their virulence ability (virulent vs. nonvirulent) are different regarding their activity and RNA affinity (Domingues *et al.*, 2009). Further studies are still necessary to confirm whether the differences observed in RNase R protein are responsible for the virulence of these strains.

In *P. syringae*, RNase R is the exoribonuclease present in the degradosome as opposed to most other systems, where PNPase is part of such complexes (Purusharth *et al.*, 2005) (see Complexes of RNases). Like in *E. coli*, RNase R is also particularly important at low temperatures, because inactivation of the *rnr* gene inhibits the growth of both *Pseudomonas putida* (Reva *et al.*, 2006) and *P. syringae* (Purusharth *et al.*, 2007) at 4 °C. In *P. syringae*, RNase R is involved in 3′ end maturation of the 16S and 5S rRNA genes and in tmRNA turnover (Purusharth *et al.*, 2007). Genomic studies revealed that *P. putida* RNase R plays an important role in mRNA turnover because its absence led to the accumulation of several mRNAs (Fonseca *et al.*, 2008). On the other hand, RNase R (previously YvaJ) from *B. subtilis* was suggested not to play a critical role in RNA degradation; however, it may play a role in mRNA turnover when polyadenylation at the 3′ end occurs (Oussenko *et al.*, 2005). Moreover, *B. subtilis* RNase R was shown to be important for the quality control of tRNAs (Campos-Guillen *et al.*, 2010).

Overall, RNase R-deficient bacteria have been shown to be less virulent than the wild-type parental strains. However, how this is achieved is still not completely clear. This is probably related to critical RNA degradation pathways. The fact that RNase R was found to be key in the degradation of sRNAs, namely the virulence regulator SsrA/tmRNA, paves the way to broaden its role in pathogenesis. It has also been suggested that RNase R may control the export of proteins involved in virulence mechanisms. Altogether, the available data suggest that bacterial RNase R may be attractive as a potential therapeutic agent, but clearly more studies are required.

Oligoribonuclease

The end products resulting from the degradation of previously described RNases constitute a severe problem to the cell viability, because these enzymes release RNA fragments of 2–5 nucleotides in length whose accumulation may be deleterious (Ghosh & Deutscher, 1999). Oligoribonuclease is the enzyme that degrades these short oligoribonucleotides (Stevens & Niyogi, 1967; Niyogi & Datta, 1975). From the known exoribonuclease genes in *E. coli* the oligoribonuclease gene, *orn*, is the only one required for cell viability (Ghosh & Deutscher, 1999).

Oligoribonuclease belongs to the DEDD family of exoribonucleases (Zuo & Deutscher, 2001), and is a homodimeric ($\alpha 2$) enzyme (Zhang *et al.*, 1998) that produces mononucleotides and requires the presence of divalent cations (Mn^{2+}) (Niyogi & Datta, 1975) (Fig. 1b). The hydrolysis is

processive in the 3′–5′ direction; this enzyme has a higher affinity to 5-mer oligoribonucleotides and the reaction rate decreases with increasing chain length (Datta & Niyogi, 1975). This enzyme requires a free 3′-OH end and is not sensitive to the 5′-phosphorylation state of the RNA (Datta & Niyogi, 1975). Only the preliminary X-ray characterization of the *E. coli* oligoribonuclease structure has been reported (Fiedler *et al.*, 2004). It was shown recently that Orn can degrade short DNA oligos, like its human homologue Sfn, but this degradation requires higher enzyme concentrations than the RNA-directed activity (Mechold *et al.*, 2006).

Bacillus subtilis does not have an oligoribonuclease (Orn) homologue. However, a functional analogue of Orn was identified in this organism that was named YtqI (NrnA). Surprisingly, this protein *in vitro* can degrade not only short oligonucleotides (with a preference for 3-mer) but also 3′-phosphoadenosine 5′-phosphate (pAp). This suggests the existence of a closer link between sulfur and RNA metabolism in *B. subtilis* (Mechold *et al.*, 2007). More recently, a second nanoRNase was discovered and named YngD (NrnB). This protein is a member of the DHH/DHHA1 protein family of phosphoesterases, and degrades nanoRNA 5-mers *in vitro* similar to oligoribonuclease from *E. coli* (Fang *et al.*, 2009).

In *Streptomyces griseus* and *S. coelicolor*, the gene *ornA* encodes the oligoribonuclease protein. It is transcribed from two promoters: one that is developmentally regulated and the other that is a constitutive promoter (Ohnishi *et al.*, 2000). Unlike *E. coli*, in which oligoribonuclease is an essential enzyme, if the *ornA* gene is deleted, the cells are viable, but not able to form aerial hyphae (Ohnishi *et al.*, 2000). It was also shown that the degradation of RNA oligomers by oligoribonuclease is critical for the completion of the life cycle (Sello & Buttner, 2008).

In RNA metabolism, oligoribonuclease acts as the 'finishing enzyme' to degrade oligoribonucleotides of two to five nucleotides in length to mononucleotides in a wide range of organisms.

RNase J1/J2

Recently, the discovery of RNase J1 and J2 shed new light on the mechanism of RNA degradation in *B. subtilis*. These enzymes were the first to be demonstrated to have bacterial 5′–3′ exoribonucleolytic activity (Mathy *et al.*, 2007). Moreover, two different activities can be observed for these enzymes, because they can act both as endo- and as exoribonucleases (Even *et al.*, 2005). RNases J1 and J2 had already been described under endoribonucleases (see the above section on Other endonucleases). RNase J1 is an essential protein (Even *et al.*, 2005) and its exoribonucleolytic activity depends on the phosphorylation state at the 5′ end, with a preference for monophosphate substrates (Mathy *et al.*, 2007). It was also shown that RNase J1 requires a single-stranded 5′ end to allow the exoribonucleolytic activity (Mathy *et al.*, 2007). It also functions as a 5′–3′ exoribonuclease in the maturation of the 16S rRNA gene and in regulating the mRNA stability of the *Bacillus thuringiensis* stationary-phase insecticidal protein transcript *cryIIIA* and the *trp* leader sequence (Mathy *et al.*, 2007; Deikus *et al.*, 2008). There are indications that RNase J1 plays an important role both in the maturation or degradation of specific RNAs and in governing global mRNA stability (Mader *et al.*, 2008). Interestingly, RNase J homologues are not present in *Gammaproteobacteria* such as *E. coli*, but are widely distributed in other bacteria and in archaea (Even *et al.*, 2005; Mathy *et al.*, 2007).

Other 3′–5′ exonucleases

In *E. coli*, besides the exoribonucleases mentioned above, three others are present in the cell: RNase PH, RNase D and RNase T.

RNase PH belongs to the same family of PNPase, the PDX family of exoribonucleases (see Fig. 1b). It is encoded by the *rph* gene and cotranscribed with pyrE, a gene necessary for pyrimidine synthesis that is located upstream of *rph* (Ost & Deutscher, 1991). However, while PNPase has an important function in mRNA degradation, RNase PH is involved in tRNA metabolism, namely in the processing of tRNA precursors (Deutscher *et al.*, 1988; Kelly *et al.*, 1992). RNase PH can act as a phosphorolytic RNase by removing nucleotides following the CCA terminus of tRNA and also as a nucleotidyltransferase by adding nucleotides to the ends of RNA molecules (Jensen *et al.*, 1992; Kelly & Deutscher, 1992). RNase PH can also cleave off the 3′ end of other sRNAs, including M1, 6S and 4.5S RNA (Li *et al.*, 1998). Deletion of the *rph* gene has no effect on the growth or the viability of the cells. However, the combination of this deletion with RNase T or PNPase deletions affects growth. These data suggest that RNase PH has overlapping functions *in vivo* with both RNase T and PNPase (Kelly *et al.*, 1992). In *B. subtilis*, there are two pathways for tRNA maturation and RNase PH seems to be the most important for the maturation of tRNA precursors with CCA motifs, while RNase Z is responsible for the processing of CCA-less tRNA precursors (Wen *et al.*, 2005). The crystal structure of *B. subtilis* RNase PH has been determined with a medium resolution and it can be superimposed to the second core domain structure of PNPase. Similar to what happens with RNase PH from *A. aeolicus* and *Pseudomonas aeruginosa*, the protein crystallizes as a hexamer arranged as a trimer of dimers and the substrate interacts with the dimer (Ishii *et al.*, 2003; Choi *et al.*, 2004; Harlow *et al.*, 2004). However, the hexameric ring formation is essential for the binding of precursor tRNA and also for exoribonucleolytic activity (Choi *et al.*,

2004). In *Streptomyces*, an RNase PH-like enzyme encoded by the *SCO2904* gene was identified. Similar to PNPase, this can polyadenylate the 3′ end of RNA *in vitro*; however, *in vivo* studies showed that RNase PH may not be involved in the synthesis or the maintenance of poly(A) tails in *S. coelicolor* (Bralley *et al.*, 2006). In *Streptomyces*, all essential tRNA genes must encode the CCA end and the RNase PH must be required to induce maturation of the 3′ end of these tRNAs (Bralley *et al.*, 2006) (see also below the section on processing).

RNase D is a 3′–5′ hydrolytic exoribonuclease from the DEDD superfamily, which contains both DNA and RNA exonucleases (Zuo & Deutscher, 2001) (Fig. 1b). As a member of this family, it has three conserved motifs. In motif III, the presence of a tyrosine or histidine led to the division of this family into two subgroups, DEDDy and DEDDh, with RNase D belonging to the first one (Zuo & Deutscher, 2001). RNase D requires divalent metal ions for its activity and has a high degree of substrate specificity; its substrates include denatured and damaged tRNAs, as well as tRNA precursors with extra 3′ residues following the CCA sequence, but not ssRNA (Cudny & Deutscher, 1980; Cudny *et al.*, 1981; Zhang & Deutscher, 1988b) (see also below the section on processing). RNase D overexpression seems to be deleterious for the cell (Zhang & Deutscher, 1988a). The chromosomal gene uses UUG as the initiation codon and has an abnormally high level of rare codons, which could limit the levels of endogenous protein (Kane, 1995). Moreover, it was shown that RNase D expression is negatively regulated at the translational level by the initiation codon (Zhang & Deutscher, 1989). The crystal structure of RNase D shows that this protein has one DEDD catalytic domain and two HRDC domains with a funnel-shaped ring architecture that could be important to define the exoribonucleolytic activity of RNase D, which may be processive (Zuo *et al.*, 2005). RNase D homologues have been found in many organisms, except archaea, and, in some genomes, it is possible to find more than one homologue (Zuo & Deutscher, 2001).

RNase T is a 3′–5′ exoribonuclease that belongs to the DEDD superfamily of RNases and to the DEDDh subgroup (Zuo & Deutscher, 2001) (Fig. 1b). It is a single-strand-specific exonuclease and the activity is dependent on the presence of divalent metal ions, such as Mg^{2+} or Mn^{2+} (Deutscher & Marlor, 1985; Zuo & Deutscher, 2002). Besides the ability to cleave RNA molecules, RNase T also has DNA exonuclease activity (Viswanathan *et al.*, 1998). RNase T has a distributive activity and an unusual base specificity, discriminating against pyrimidines and, particularly, C residues (Zuo & Deutscher, 2002). This sequence specificity is largely determined by the last four nucleotides at the 3′ end (Zuo & Deutscher, 2002). It is involved in the final step of maturation of many stable RNAs and seems to be the most important RNase with that function (Li & Deutscher, 1995, 1996; Li *et al.*, 1998). In fact, it was shown that RNase T is essential for the maturation of the 3′ ends of 5S and 23S rRNA genes (Li & Deutscher, 1995; Li *et al.*, 1999a), and it is also involved in the end turnover of tRNAs (Deutscher *et al.*, 1985). The crystal structures of RNase T from both *E. coli* and *P. aeruginosa* show that the protein adopts an oligoribonuclease-like homodimer architecture, which was shown to be required for its activity (Li *et al.*, 1996; Zuo *et al.*, 2007). The two monomers are facing opposite ends, which means that the active site of one monomer is facing the binding site of the other. This arrangement allows the binding of the RNA molecule from one monomer to be close to the active site of the other one (Zuo *et al.*, 2007). Despite its critical role in RNA metabolism, RNase T orthologues are just found in a small group of bacteria, the *Gamma* division of *Proteobacteria* (Zuo & Deutscher, 2001).

Both *E. coli* and *Salmonella* belong to the *Enterobacteriaceae* family. A recent work showed that the two hydrolytic enzymes present in *E. coli*, RNase II and RNase R, are also found in *Salmonella* and behave quite similarly in terms of their the ability to degrade structured substrates and the final product that is released. However, the proteins from *Salmonella* showed a reduction in their activity and an ability to bind to RNA when compared with the *E. coli* enzymes (Domingues *et al.*, 2009).

In *B. subtilis*, besides the proteins mentioned above, we can find other RNase, YhaM. This protein has been implicated in DNA replication (is able to degrade ssDNA), and *in vitro* studies showed that is also able to cleave RNA into the 3′–5′ direction in a Mn^{2+}-dependent manner. However, the *in vivo* function of YhaM in RNA metabolism remains to be determined (Noirot-Gros *et al.*, 2002; Oussenko *et al.*, 2002). Sequence homologues of YhaM were found only in Gram-positive bacteria (Oussenko *et al.*, 2002).

Cyanobacteria are prokaryotes organisms that may be related to the ancestor of chloroplasts. In the genome of *Synechocystis*, it is possible to find genes that have a high homology to RNase E, PNPase, RNase II/R and PAP, the most important proteins involved in mRNA degradation and polyadenylation (Rott *et al.*, 2003). However, the product of the putative PAP gene has nucleotidyltransferase and not PAP activity, and the reaction of polyadenylation in *Synechocystis* is performed by PNPase, which originates heterogeneous poly(A)-rich tails, like it occurs in chloroplasts. These tails are found in the amino acid coding region, the 5′ and 3′ untranslated regions of mRNAs, in rRNA and the single intron located at the tRNAfmet (Rott *et al.*, 2003). PNPase is an essential protein for this organism because the deletion of this gene causes lethality. The same is observed when the gene for RNase II/R is disrupted (Rott *et al.*, 2003). There is no degradosome complex in cyanobacteria (see Complexes of RNases).

Complexes of RNases

RNA-degrading machines

The degradosome is a large multiprotein complex involved in RNA degradation. It is believed to act as a general RNA decay machine in which the components of the degradosome cooperate during the decay of many RNAs. The complex formation contributes to the coordination of the endoribonucleolytic cleavage with the exoribonucleolytic degradation (Py *et al.*, 1994, 1996; Miczak *et al.*, 1996; Vanzo *et al.*, 1998).

In *E. coli*, this multiprotein complex is formed by RNA degradation enzymes RNase E and the exonuclease PNPase, as well as the ATP-dependent RhlB and the glycolytic enzyme enolase (Py *et al.*, 1994; Miczak *et al.*, 1996; Vanzo *et al.*, 1998). RNase E provides the organizing scaffold for the degradosome, through its carboxy-terminal half. In the carboxy-terminal half, four segments were found to show a tendency to form a secondary structure (Callaghan *et al.*, 2004), namely A, B, C and D. Segment A localizes the degradosome to the inner cytoplasmic membrane (Khemici *et al.*, 2008). RhlB binds a 69-residue conserved segment downstream of segment B, a coiled coil that may engage RNA (Chandran *et al.*, 2007; Worrall *et al.*, 2008b). Segment C is the enolase-binding site (Chandran & Luisi, 2006), and segment D interacts with PNPase (Callaghan *et al.*, 2004).

Under normal growth conditions, crystallographic and biophysical measurements indicate that one enolase dimer and one helicase protomer interact with one RNase E monomer (Chandran & Luisi, 2006; Chandran *et al.*, 2007; Worrall *et al.*, 2008a). Findings for the stoichiometry of PNPase with the isolated recognition site from RNase E (Callaghan *et al.*, 2004), and recent crystallographic analysis of the *E. coli* PNPase/RNase E complex reveal an equimolar ratio (Nurmohamed *et al.*, 2009). In principle, three RNase E tetramers and four PNPase trimers could form a self-closing assembly composed of 12 protomers, satisfying all possible binding sites. The ideal composition of such an assembly is 12 : 12 : 24 : 12 (RNase E : PNPase : enolase : RhlB) (Marcaida *et al.*, 2006).

The group of minor components that bind to the degradosome to affect its composition and modulate its enzymatic activity includes polyphosphate kinase, poly(A) polymerase, ribosomal proteins and the molecular chaperones DnaK and GroEL (Miczak *et al.*, 1996; Butland *et al.*, 2005; Morita *et al.*, 2005; Regonesi *et al.*, 2006) and other DEAD-box helicases (SrmB, RhlE and CsdA) that may bind to sites outside the RhlB recognition region (Khemici & Carpousis, 2004). Another potential interaction may occur between the degradosome and the cytoskeleton protein MinD (a membrane-localized bacterial cytoskeletal protein), which may account for the apparent association of the degradosome with the cytoskeleton (Taghbalout & Rothfield, 2007).

The composition of the degradosome can also undergo changes depending on the conditions of growth or stress (Khemici *et al.*, 2004; Prud'homme-Genereux *et al.*, 2004; Morita *et al.*, 2005; Gao *et al.*, 2006). A different complex containing RNase E, Hfq and SgrS, a small regulatory RNA, is formed under conditions of phosphosugar stress (Morita *et al.*, 2005). The formation of the complex with Hfq and SgrS requires the same region of RNase E that is necessary for the formation of the canonical RNA degradosome, and evidence suggests that the degradosome is remodelled as a consequence of the new interaction. There is evidence that RNase E can form a 'cold-shock' RNA degradosome in which the helicase RhlB is replaced by CsdA, another DEAD-box RNA helicase (Khemici *et al.*, 2004; Prud'homme-Genereux *et al.*, 2004). The compositional changes in the degradosome following cold exposure may account, in part, for changes in mRNA stability associated with cold shock response. The PNPase content of the degradosome can change in response to phosphosugar stress, temperature shock and the growth stage (Beran & Simons, 2001; Liou *et al.*, 2001). Surprisingly, RNase E from *P. syringae* interacts with the hydrolytic exoribonuclease RNase R instead of PNPase and with another DEAD-box helicase, RhlE (Purusharth *et al.*, 2005).

Degradosome composition and function may also be modulated through its interactions with the RNase E inhibitory proteins RraA and RraB, which interact with the C-terminal half of RNase E, thereby altering the composition of the degradosome, namely the amount of PNPase, RhlB and enolase bound to RNase E. RraB expression gave rise to degradosomes that contained the noncanonical components DnaK and CsdA.

The global effects of mutations in degradosome constituents on mRNA levels have been evaluated using microarrays (Bernstein *et al.*, 2004). This work reported that the functions of all degradosome constituents are necessary for normal mRNA turnover and that assembled degradosome components work in concert to regulate the transcripts of some *E. coli* metabolic pathways, but not others. This suggests the existence of structural features or biochemical factors that distinguish among different classes of mRNAs targeted for degradation.

Archaea are microscopic, single-celled organisms with no nucleus, no mitochondria and no chloroplasts. Regarding mRNA, they are more similar to bacteria than to eukaryotes: mRNA does not have introns, it is polycistronic, is not modified and does not have long stabilizing poly(A) tails at the 3′ end (Brown & Coleman, 1975; Brown & Reeve, 1986). However, in *Sulfolobus* and *Methanothermobacter*, the existence of an archaeal exosome with characteristics of the eukaryotic exosome was demonstrated (Evguenieva-Hackenberg *et al.*, 2003; Farhoud *et al.*, 2005). The exosome is a multiprotein complex involved in the maintenance of the

correct levels of mRNA in eukaryotic cells (van Hoof & Parker, 1999) (see also below the section on RNA degradation on eukaryotic microorganisms). The exosome of the archaeon *S. solfataricus* is a protein complex with a dual function: it is an RNA-tailing and RNA-degrading enzyme because it has both phosphorolytic and polyadenylating activity (Lorentzen *et al.*, 2005; Portnoy *et al.*, 2005). It is formed by a hexameric ring consisting of three dimers of the orthologues of Rrp41 and Rrp42, and is responsible for phosphorolytic RNA degradation (Lorentzen *et al.*, 2005). It is able to synthesize heteropolymeric RNA tails, and, generally, RNA synthesis by the hexameric ring is more efficient than RNA phosphorolysis (Evguenieva-Hackenberg *et al.*, 2008). The Rrp41 orthologue contains the active site; however, the ring structure is necessary for the activity of the complex (Lorentzen *et al.*, 2005). On the top of the ring there are three polypeptides with RNA-binding domains that are orthologues of Rrp4 (which contains S1 and KH domains) and/or Csl4 (which contains S1 and Zn-ribbon domains) (Buttner *et al.*, 2005; Lorentzen *et al.*, 2007). Recently, the structure of the *S. solfataricus* exosome was resolved (Lu *et al.*, 2010). The structure showed that the RNA-binding ring is flexible, which may be important for the unwinding of secondary structures (Lu *et al.*, 2010). The structure of the archaeal nine-subunit exosome is very similar to the one present in Eukarya and to PNPase (Lorentzen *et al.*, 2005, 2007; Liu *et al.*, 2006b). However, the archaeal exosome contains at least one additional subunit with an unknown function, a protein designated DnaG (Evguenieva-Hackenberg *et al.*, 2003), which can participate in 5S rRNA gene maturation. The *S. solfataricus* exosome is able to degrade synthetic and natural RNA efficiently, which is in accordance with its proposed role as a major complex of 3′ to 5′ exoribonucleases in the cell. Moreover, the genome of *S. solfataricus* does not contain genes for other predicted 3′–5′ exoribonucleases. In the absence of triphosphate at the 5′ end, the mRNA degradation can also occur in the 5′–3′ direction (Hasenohrl *et al.*, 2008). In this case, the degradation is probably performed by the RNase J1/J2 homologue, which is identical to the Mbl-like RNase (Koonin *et al.*, 2001).

However, in halophilic and many methanogenic archaea genomes, it is not possible to find the orthologues of exosomal subunits, which indicates that the mechanism for RNA degradation may be different in these archaea (Koonin *et al.*, 2001). Moreover, in archaea without an exosome, there is no post-transcriptional modification of the RNA molecules, and no tails are added to RNAs (Portnoy *et al.*, 2005; Portnoy & Schuster, 2006). In halophilic archaea, there is an RNase R-like protein that is not found in methanogenic archaea (Portnoy & Schuster, 2006). Like in *Mycoplasma*, these archaea also have a minimal genome, and, for this reason, the RNase R homologue may be the only enzyme responsible for the exoribonucleolytic activity, because both exosome and PNPase are absent (Zuo & Deutscher, 2001). *Haloferax volcanii* is a representative halophilic archaeon. It was shown that RNase R is required for viability in *H. volcanni*, and therefore, plays an important role in the mechanism of RNA degradation independent of polyadenylation (Portnoy *et al.*, 2005; Portnoy & Schuster, 2006).

The RNases in action

Processing and degradation of RNAs

Processing of RNAs

All rRNA and tRNA species are transcribed as precursor molecules that further undergo a series of modifications to achieve the mature molecules (Deutscher, 2009). Here, we will focus on the importance of RNases in the processing events during the maturation of rRNA and tRNA effectors. We will also refer to their role in the quality control of these processes.

In prokaryotes, the 70S ribosomes are constituted of two subunits: 30S and 50S particles. The smaller subunit comprises a 16S rRNA molecule and 21 proteins, and the larger subunit comprises a 23S and a 5S rRNA molecules plus 33 proteins. rRNAs are transcribed as precursor molecules that are processed and modified while assembly is occurring. In *E. coli*, there are seven rRNA operons comprising the three rRNA molecules always displayed in the same order: the 16S gene at the 5′ end, followed by the 23S, and finally by the 5S rRNA gene at the 3′ end (Deutscher, 2009). During transcription, RNase III cleaves double-stranded structures in the pre-rRNAs, releasing the fragments that will be subsequently cleaved to generate the 16S, 23S and 5S rRNA genes (Robertson *et al.*, 1968; Gegenheimer & Apirion, 1975).

RNase E further reduces the extra 115 nt from the 17S rRNA gene (16S rRNA gene precursor) to 66 at the 5′ end, resulting in a 16.3S intermediate. Finally, RNase G (also termed RNase M16) converts the 5′ end to the mature molecule (Hayes & Vasseur, 1976; Dahlberg *et al.*, 1978; Li *et al.*, 1999b). In *B. subtilis*, the 5′–3′ exoribonuclease RNase J1 is involved in rRNA processing (Even *et al.*, 2005; Britton *et al.*, 2007; de la Sierra-Gallay *et al.*, 2008). The 3′ maturation enzyme remains to be characterized both in *E. coli* and in *B. subtilis*. In *P. syringae* the 3′–5′ exonuclease RNase R seems to be acting to directly induce the maturation of the 3′ terminus of the 16S rRNA gene (Cheng & Deutscher, 2002, 2005; Deutscher, 2006, 2009; Purusharth *et al.*, 2007).

The *E. coli* 23S rRNA gene precursor is released, harboring three or seven 5′ and seven to nine 3′ extra residues. The 3′ maturation requires RNase T for completion (Li *et al.*, 1999a). In *B. subtilis* the RNase III family Mini-III dimeric enzyme is responsible for the simultaneous maturation of both 5′ and 3′ sides of the double-stranded stalk that flanks

the mature 23S rRNA gene (Olmedo & Guzman, 2008; Redko *et al.*, 2008). *Salmonella* constitutes an interesting case where RNase III removes IVS in a way that the mature rRNA molecule results from two fragments (Burgin *et al.*, 1990).

The *E. coli* 5S rRNA gene derives from a 9S precursor, which is endonucleolytically cleaved by RNase E, releasing an intermediate molecule with three additional nucleotides at both ends (Ghora & Apirion, 1978; Misra & Apirion, 1979). The 5′ maturation is still uncharacterized, while RNase T is again responsible for removing (at least) the least two 3′ residues (Li & Deutscher, 1995). *Bacillus subtilis* almost repeats the mechanism of maturation of the 23S for the 5S rRNA gene, but in this case, RNase M5 cleaves the double-stranded region, simultaneously inducing the maturation of the 5′ and 3′ ends (Sogin *et al.*, 1977).

rRNA degradation takes place whenever errors (e.g. improper structure conformations, or misordered addition of proteins) occur and also in response to stress conditions (Deutscher, 2009). Quality control mechanisms occur at levels that are almost negligible in fast-growing cells, but are nevertheless essential as they avoid the accumulation of defective ribosomes. RNase LS may participate in the 23S rRNA gene degradation; PNPase, together with an RNA helicase or RNase R, may also be involved, because they are the only ones that can degrade structured RNAs. In addition to these, any process that leads to damaged cell membranes induces drastic RNA degradation, because it promotes the release of the nonspecific endoribonuclease RNase I from the periplasm into the cells (Cheng & Deutscher, 2005; Otsuka & Yonesaki, 2005; Deutscher, 2009).

tRNAs are vital adaptors for the decoding of the genome into proteins, and contribute up to 20% of the total RNA in the cell (Dittmar *et al.*, 2004; Hartmann *et al.*, 2009). Both *E. coli* K12 and *B. subtilis* bear 86 tRNA genes in their genome, many of them associated into operons (Fournier & Ozeki, 1985; Inokuchi & Yamao, 1995; Dittmar *et al.*, 2004). Introns are rarely found and are present only in the anticodon loop of some tRNAs in bacteria, but occur extensively in archaea (Vogel & Hess, 2001; Marck & Grosjean, 2002, 2003). Two endoribonucleases mainly process the pre-tRNAs: RNase P, which almost universally generates 5′ mature ends (Evans *et al.*, 2006; Randau *et al.*, 2008), and RNase Z, which cleaves the CCA-less pre-tRNAs (see the sections on RNase P and RNase Z for details on these enzymes). All tRNA molecules must have a CCA signal at their 3′ end to allow aminoacylation by the tRNA nucleotidyltransferase. That can be achieved, either by removing all extra nucleotides, when it is already present in the sequence, or cutting after the discriminator nucleotide (Li & Deutscher, 1995; Hartmann *et al.*, 2009). The CCA motif varies from absent in eukarya to being present in all genes of *E. coli*, about 2/3 of the *B. subtilis* pre-tRNAs, and from 0% to 100% in archaea (Hartmann *et al.*, 2009). Two main modes of 3′ maturation have been described so far: a one-step endonucleolytic cleavage by the universally conserved RNase Z homodimer (Dutta & Deutscher, 2009) and a multistep process involving both endo- and exonucleases (Li *et al.*, 1998; Hartmann *et al.*, 2009).

For instance, in *E. coli* where all genes encode the CCA sequence, maturation usually begins with an RNase E cut at the 3′ end (eventually aided by PNPase or RNase II), followed by 5′ processing by RNase P, and a final 3′ exonucleolytic trimming to expose the CCA sequence. The trimming reaction is carried out by RNase II, RNase D, or more effectively, RNase T or RNase PH (Li & Deutscher, 2002; Ow & Kushner, 2002).

Even though RNase Z is not essential for *E. coli*, it is encoded in its genome and has been shown to be able to shut down growth when overexpressed (Takaku & Nashimoto, 2008).

In *B. subtilis* all the CCA-less tRNAs are processed by the RNase Z and all the CCA-containing tRNAs are envisaged to follow a multistep maturation pathway, although the endonuclease responsible for the first step has not yet been found (Pellegrini *et al.*, 2003). RNase PH is the main exo involved in the trimming process (Wen *et al.*, 2005).

tRNAs have several constraints because they must be sufficiently similar to be processed, and able to fit within the ribosome, but must be sufficiently different to ensure correct loading with specific amino acids and recognize exclusively the codon(s) for their anticodon sequence (Hopper *et al.*, 2010). Modifications are of absolute importance for folding stabilization avoiding rapid decay, fidelity and efficiency of aminoacylation and/or proper binding to the ribosomes (Hou & Perona, 2010; Phizicky & Alfonzo, 2010). Indeed, about 100 modifications have been described for tRNAs so far (Czerwoniec *et al.*, 2009; Hopper *et al.*, 2010). Although tRNAs are stable, they have quality control mechanisms for eliminating defective species, and it seems at least partially dependent on polyadenylation by poly(A) polymerase (and removal by polynucleotide phosphorylase). RNase R has also been shown to participate in tRNA quality control mechanisms in a *B. subtilis* conditional CCA mutant strain. In this sense, flawed stable RNA molecules would behave like unstable RNAs being rapidly degraded by similar mechanisms (Li *et al.*, 2002; Campos-Guillen *et al.*, 2010).

tmRNA is a hybrid/bifunctional RNA molecule that shares the characteristics of both tRNA structural folds involving the 3′ and 5′ ends (Hayes & Keiler, 2010) – and mRNA – bearing a sequence that encodes for an ORF, consisting of a peptide signal for proteolytic degradation, ended with UAA termination codons. The tmRNA maturation is similar to the mechanism described above regarding tRNA processing. However, it was shown that RNase R is quite important for the maturation of the 3′ end of the

tmRNA, even more relevantly under cold-shock conditions (Cairrão *et al.*, 2003). SmpB is a small basic protein that binds to tmRNA with a high affinity and specificity (Karzai *et al.*, 1999; Dulebohn *et al.*, 2006), and specifically recognizes paused ribosomes near the 3′ end of truncated mRNAs (Janssen & Hayes, 2009). This RNA-binding protein is a regulator for the tmRNA-based quality control system in the cells, because it can prevent tmRNA degradation by RNase R (Hong *et al.*, 2005).

RNA degradation mechanisms

The same RNA molecule can be degraded by different pathways depending on the stress conditions or the growth phase. Thus, the degradation pathways are not universal. However, the interplay between the different factors involved in RNA decay emphasizes the role of RNases in the degradation of multiple substrates (Fig. 3).

In this section, we illustrate various examples of the relevant mechanisms of mRNAs and sRNAs degradation mainly in *E. coli*, but we also refer to examples from *B. subtilis*.

pyrF-orfF

The dicistronic transcript from *pyrF-orfF* contains *pyrF*, encoding orotidine-5′-monophosphatase decarboxylase, and an ORF (*orfF*) encoding a polypeptide of unknown function (Donovan & Kushner, 1983; Jensen *et al.*, 1984; Turnbough *et al.*, 1987). The full-length transcript is rapidly cleaved into a series of breakdown products, and at least 18 endonucleolytic cleavage sites have been mapped throughout the full-length mRNA (Arraiano *et al.*, 1997). Moreover, it seems that the *pyrF-orfF* transcript may be degraded by more than one enzymatic pathway depending on where the initial cleavage occurs. Therefore, some fragments seem to be degraded in a 5′–3′ direction, while other degradation products are processively cleaved in a 3′–5′ direction. The results obtained by Arraiano *et al.* (1997) provided, for the first time, support to the hypothesis that multiple decay pathways are involved in the decay of a single transcript. It thus seems reasonable to assume that *in vivo* there are a variety of ways in which a particular mRNA can be degraded. Which pathway is used may be related to the particular context in which one or more of the decay-mediating factors has access to the mRNA.

trxA

The *E. coli trxA* gene, which encodes for thioredoxin, is transcribed as a monocistronic message of 493 nucleotides. In the study of the *trxA* decay multiple mutant strains were constructed deficient in RNase E (*rne* – previously known as *ams*), PNPase (*pnp*) and RNase II (*rnb*) (Arraiano *et al.*, 1988). Northern and S1 analysis showed that full-length transcripts are initially processed by endonucleolytic cleavages (Arraiano *et al.*, 1993). The complete degradation of the initially cleaved transcripts occurs through progression of endonucleolytic steps in the 3′–5′ direction, followed by exonucleolytic degradation by RNase II and PNPase. This was the first report of a progression of endonucleolytic cleavages in a 3′–5′ direction during the degradation of a full-length transcript.

rpsO

The *rpsO* gene encodes for the *E. coli* ribosomal protein S15. The degradation of *rpsO* mRNA is accomplished by several independent pathways, including the RNase E-dependent endonucleolytic pathway and a pathway that requires the polyadenylation of transcripts (Braun *et al.*, 1996). The stability of the *rpsO* transcript is mainly controlled by RNase E. After RNase E cleavage, the mRNA lacking the 3′-terminal RNA secondary structure becomes an ideal substrate for PNPase (Braun *et al.*, 1996). When the primary pathway of decay mediated by RNase E is inactive, the exoribonucleolytic poly(A)-dependent degradation of *rpsO* mRNA is stimulated (Hajnsdorf *et al.*, 1995; Marujo *et al.*, 2003; Folichon *et al.*, 2005). It was shown that RNase R is the main enzyme involved in the poly(A)-dependent degradation of the *rpsO* mRNA (Andrade *et al.*, 2009a) and that RNase II protects the full-length *rpsO* mRNA from degradation by removing the poly(A) tails (Marujo *et al.*, 2000). Elongated *rpsO* transcripts harboring poly(A) tails of increased length are specifically recognized by RNase R and strongly accumulate in the absence of this exonuclease. Because this enzyme is able to degrade dsRNAs, the 3′ oligo(A)-extension may stimulate the binding of RNase R, allowing the complete degradation of the *rpsO* mRNA. The RNA chaperone Hfq can protect the *rpsO* mRNA from exonucleolytic degradation by PNPase and RNase II, and from cleavage by RNase E (Folichon *et al.*, 2003). Moreover, it was shown recently that in the absence of this chaperone, stabilization of *rpsO* mRNA occurs, with a concomitant decrease in its level, indicating that the change in the mRNA levels in the *hfq* mutant does not result from the modification of RNA stability, but probably from changes in transcriptional activity (Le Derout *et al.*, 2010).

rpsT

The *rps*T gene encodes the *E. coli* ribosomal protein S20. This gene is transcribed from two promoters (P1 and P2) and terminates at a Rho-independent terminator, yielding two monocistronic mRNA species: P1 (447 nt) and P2 (356 nt) (Mackie & Parsons, 1983). The first step of the *rpsT* decay is carried out by RNase E and there are several lines of evidence indicating that this step is independent of polyadenylation (Mackie, 1991; Coburn & Mackie, 1996b, 1998). However,

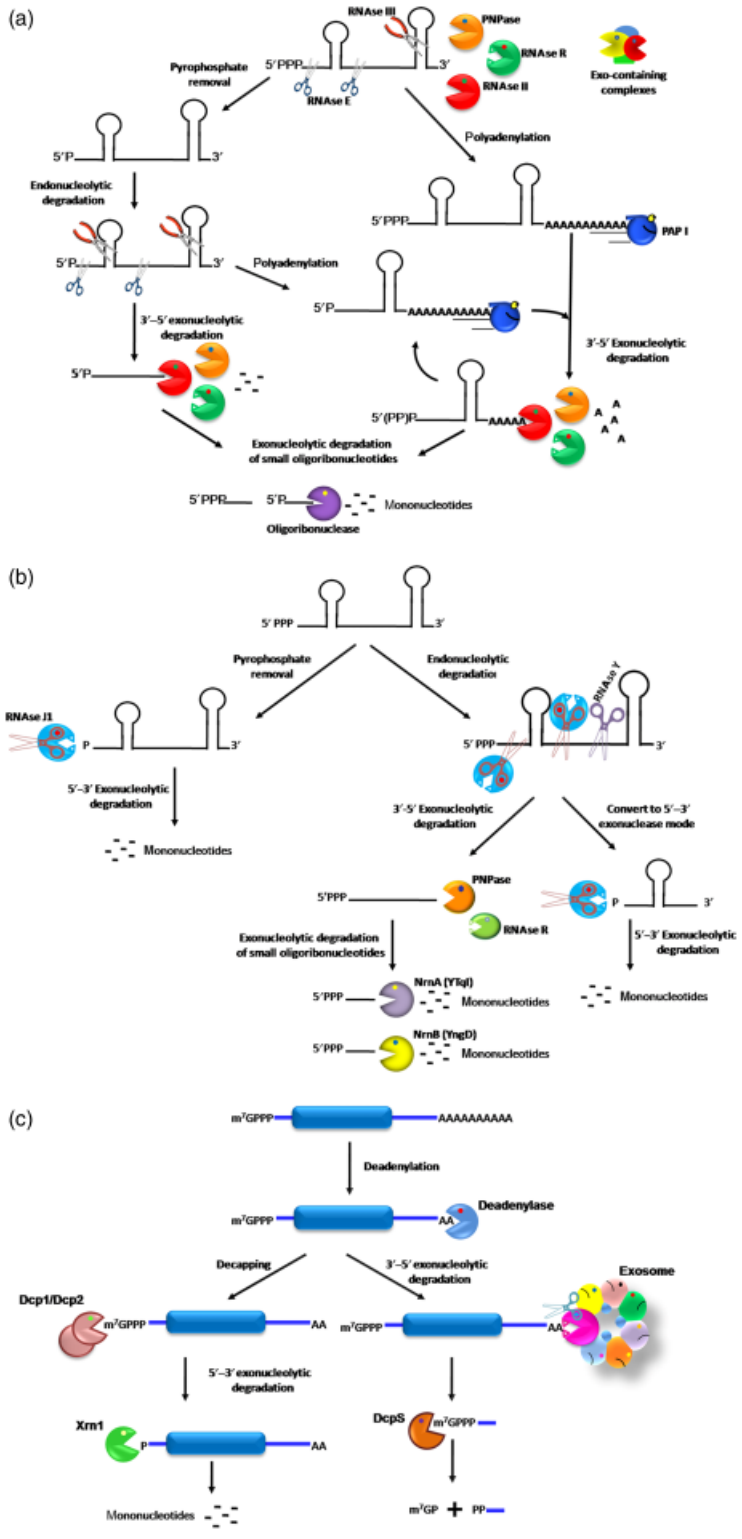

Fig. 3. Mechanisms of decay. (a) Model of RNA degradation pathways in *Escherichia coli*. The decay of the majority of transcripts starts with an endoribonucleolytic cleavage by RNase E. This endoribonuclease prefers a monophosphorylated 5′ end, but not in a strict way, and several RNAs escaping this rule have been described (Kime *et al.*, 2009). RNase III is another enzyme responsible for the initial endoribonucleolytic cleavage of structured RNAs. However, unlike RNase E (that only cleaves single-stranded RNAs), RNase III cleaves dsRNAs. After endoribonucleolytic cleavages, the linear transcripts are rapidly degraded by the 3′–5′ degradative exoribonucleases, RNase II, RNase R and PNPase. RNase R, unlike RNase II and PNPase, is efficient against highly structured RNAs. PNPase, in association with other proteins, namely RNA helicases, can also unwind RNA duplexes. A minor pathway in the cell is the exoribonucleolytic degradation of full-length transcripts. Poly(A) polymerase (PAP I) adds a poly(A) tail to the short 3′ overhang. These tails provide a 'toe-hold' to which exoribonucleases can bind. Cycles of polyadenylation and exoribonucleolytic digestion can overcome RNA secondary structures. The small oligoribonucleotides (two to five nucleotides) released by exoribonucleases are finally degraded to mononucleotides by oligoribonuclease (Andrade *et al.*, 2009b). (b) Model of RNA degradation pathways in *Bacillus subtilis*. In *B. subtilis*, the main enzyme responsible for RNA decay is RNase J1. RNase J1 has both an endoribonucleolytic and a 5′–3′ exoribonucleolytic activity (Mathy *et al.*, 2007). RNase J2 has endoribonucleolytic cleavage activities and specificities similar to RNase J1 and normally they form a complex. RNAs can be degraded from the 5′ end by the 5′–3′ exoribonuclease activity of RNase J1, or first, they can be endonucleolytically cleaved by RNase J1 or by RNase Y (Shahbabian *et al.*, 2009). The products from this endoribonucleolytic cleavage can then be degraded by the 3′–5′ exoribonucleases, PNPase and RNase R, or by the 5′–3′ exoribonuclease activity of RNase J1 (Bechhofer, 2009). The small oligoribonucleotides released by the 3′–5′ exoribonucleases are finally degraded to mononucleotides by the NrnA (YTqI) or the NrnB (YngD) enzymes (Fang *et al.*, 2009). (c) Model of RNA degradation in eukaryotes. In yeast, the mRNA decay is initiated with the shortening of the poly(A) tail at the 3′ end (deadenylation). After deadenylation, there are two possible degradation pathways for the transcripts. One is the removal of the 5′ cap structure of the transcripts by the Dcp1p/Dcp2p decapping complex, leaving the RNA molecule accessible to the Xrn1 5′–3′ exoribonuclease, which rapidly degrades the uncapped RNA. The other pathway is the 3′–5′ exoribonucleolytic degradation by the exosome, a multiprotein complex in which the Rrp44 is the only active RNase (Houseley & Tollervey, 2009). Recently, it was demonstrated that Rrp44 can degrade RNA in both an exo- and an endoribonucleolytic manner (Schaeffer *et al.*, 2009). The capped oligonucleotides produced from the exosome RNA decay are hydrolyzed by the DcpS scavenger decapping enzyme (Liu & Kiledjian, 2006a).

PAP I, PNPase, ATP and phosphate are necessary to catalyze the degradation of the smaller intermediates generated by RNase E cleavage (Coburn & Mackie, 1998). On the other hand, RNase II inhibits PNPase-mediated degradation of transcripts by removing the poly(A) tails added by PAP I. The same had also been observed with *rpsO* (Coburn & Mackie,

1998; Marujo *et al.*, 2000). Therefore, RNase II paradoxically protects these RNAs from degradation by PNPase.

malEF

The polycistronic malEFG operon of *E. coli* encodes three proteins involved in the transport of maltodextrins. The *malEF* intercistronic region contains two REP sequences (Newbury *et al.*, 1987) that protect the transcript from 3′–5′ exonucleolytic degradation (Higgins *et al.*, 1988). RNase R and PNPase are shown to play a major role in the degradation of the sRNA fragments resulting from the RNase E cleavage (Khemici & Carpousis, 2004; Cheng & Deutscher, 2005). PNPase degradation of the *malEF* transcript is only accomplished in the presence of RNase E and RhlB, indicating that the degradosome complex participates in this degradation (Stickney *et al.*, 2005). RhlB unwinds the folded RNA and passes it to PNPase (Coburn *et al.*, 1999; Khemici & Carpousis, 2004). Polyadenylation of the *malEF* REP sequences by PAP I seems to be a crucial factor in the degradation of these sequences because they accumulate to high levels in *pcn*B mutants (Khemici & Carpousis, 2004).

ompA

The *ompA* gene is transcribed as a monocistronic mRNA and encodes the major protein of *E. coli* outer membrane OmpA (von Gabain *et al.*, 1983). It was demonstrated previously that *ompA* stability is growth rate dependent and that shorter generation times in the exponential phase corresponded to longer *ompA* mRNA half-lives (Nilsson *et al.*, 1984). The degradation of this mRNA is initiated by an RNase E cleavage in the 5′ UTR stem-loops (Melefors & von Gabain, 1988; Arnold *et al.*, 1998). Then, exonucleolytic degradation and polyadenylation seem to account for the elimination of breakdown products (O'Hara *et al.*, 1995; Mohanty & Kushner, 1999; Andrade *et al.*, 2006). The presence of only one of the exoribonucleases (RNase II, RNase R or PNPase) may be sufficient to remove most of the decay intermediates (Cheng & Deutscher, 2005). Furthermore, the exonucleolytic activity on the full-length *ompA* transcript was shown to be growth phase regulated (Andrade *et al.*, 2006). The sRNA MicA, first known as SraD, is the principal post-transcriptional regulator of the *ompA* expression (Rasmussen *et al.*, 2005; Udekwu *et al.*, 2005). This antisense sRNA, when present in high levels, blocks ribosome binding at the *ompA* mRNA translation start site and subsequently destabilizes this mRNA. Moreover, the MicA-mediated decay of *ompA* mRNA depends on Hfq (Rasmussen *et al.*, 2005; Udekwu *et al.*, 2005). Therefore, the levels of *ompA* are also dependent on the levels of MicA. Because OmpA is one of the main outer membrane proteins in *E. coli*, it is fundamental to have a strict regulation in order to maintain the homeostasis of the cell.

pac

Penicillin amidase, encoded by the *pac* gene, is an important enzyme for industry because it is used in the production of semi-synthetic penicillins. The degradation of this mRNA seems to be initiated by an endonucleolytic cleavage because the most remarkable stabilization of the *E. coli pac* mRNA was obtained in the RNase E mutant. RNase III seems to play no role in the degradation of this transcript. The RNase E cleavage is followed by the exonucleolytic degradation by RNase II, RNase R and/or PNPase. Single deletions of any of these exoribonucleases were unable to stabilize this mRNA most probably because of their redundant effect (Viegas *et al.*, 2005).

trp

In the last few years, the degradation of the *B. subtilis* tryptophan operon, *trp*, has been studied in detail. This operon was used recently for the study of the cleavage specificity of the RNase J1 endonuclease (Deikus & Bechhofer, 2009). The *trp* operon is regulated at the level of transcription termination (Babitzke & Gollnick, 2001; Henkin & Yanofsky, 2002), which is controlled by binding of the *trp* RNA-binding attenuation protein (TRAP) to the *trp* leader RNA. When the supply of intracellular tryptophan is low, the *trp* operon genes are transcribed from a constitutive promoter and more tryptophan is generated. When the intracellular supply of tryptophan is sufficient, the TRAP protein complex binds to a specific region of the *trp* leader sequence. This binding results in the formation of a stem-loop structure that induces transcription termination, generating a 140 nt *trp* leader RNA.

The degradation of this *trp* leader RNA is initiated by an RNase J1 endonucleolytic cleavage at a single-stranded AU-rich region upstream of the 3′ transcription terminator (Deikus *et al.*, 2008). This cleavage is followed by a 5′–3′ degradation of the downstream fragment by the exonucleolytic activity of the RNase J1 (Deikus *et al.*, 2008) and a 3′–5′ degradation of the upstream fragment by PNPase (Deikus *et al.*, 2004). The PNPase action is essential for the efficient release and recycling of TRAP (Deikus *et al.*, 2004).

sRNAs

RNases also play a very important role in the regulation of sRNAs. These RNAs have received considerable attention over the past decade because they can be crucial for the post-transcriptional control of gene expression (Storz *et al.*, 2004; Viegas & Arraiano, 2008). In order to understand the action of these sRNAs, it is fundamental to study the processing and turnover of these molecules.

sRNA MicA and RybB are stationary-phase regulators and belong to the group of sRNAs that control outer membrane permeability. RybB controls the expression of outer membrane proteins OmpC and OmpW (Guillier

et al., 2006; Johansen *et al.*, 2006) and MicA controls the expression of OmpA (Rasmussen *et al.*, 2005; Udekwu *et al.*, 2005). In *E. coli*, MicA and RybB are destabilized by PNPase in the stationary phase (Andrade & Arraiano, 2008). Moreover, PNPase can degrade MicA in a degradosome-independent manner. Polyadenylation of MicA by PAP I appears not to be essential for PNPase action on this sRNA. The 3′ exoribonucleases RNase II and RNase R appear not to be required for the degradation of MicA.

In *S. typhimurium*, the sRNAs MicA, SraL, CsrB and CsrC are also mainly degraded by PNPase in the late stationary phase. In the case of CsrB and CsrC, the absence of this exoribonuclease also induced a change in degradation patterns with the accumulation of several decay intermediates (Viegas *et al.*, 2007).

The antisense RNA CopA inhibits the replication of plasmid R1 by binding to the target region, CopT, that is located within the *repA* mRNA. This binding blocks the synthesis of the replication initiator protein RepA (Stougaard *et al.*, 1981; Givskov & Molin, 1984). The decay of CopA is initiated by an endonucleolytic cleavage by RNase E, followed by the addition of a poly(A) tail. The poly(A) tails facilitate degradation by PNPase and RNase II (Söderbom *et al.*, 1997). Both PNPase and RNase II were able to degrade the processed transcript (Söderbom & Wagner, 1998).

ColE1 RNAI is the copy number regulator of the plasmid ColE1 (Lin-Chao & Cohen, 1991). PNPase, PAP I, RNase E and RNase III have been demonstrated to play roles in ColE1 RNAI decay (Lin-Chao & Cohen, 1991; Xu *et al.*, 1993; Xu & Cohen, 1995; Binnie *et al.*, 1999). Two degradation pathways have been suggested for this RNA (Binnie *et al.*, 1999). The primary pathway starts with RNase E cleavage, followed by PAP I polyadenylation and PNPase-mediated degradation. The second mechanism begins with the polyadenylation of RNAI, followed by RNase III cleavage and a subsequent exonucleolytic attack. In the absence of RNase E, RNase III and PAP I, the antisense RNAI continues to disappear, showing that yet other enzymes are able to catalyze its decay.

The replication of the ColE2 plasmid requires a plasmid-coded initiator protein, Rep. ColE2 RNAI controls *rep* expression by the blockage of translation (Takechi *et al.*, 1994). ColE2 RNAI degradation starts with RNase E cleavage at the 5′ end. PAP I polyadenylates the 3′ ends of degradation intermediates and both RNase II and PNPase act in further exoribonucleolytic degradation (Nishio & Itoh, 2008). Because PNPase and RNase II prefer a single-stranded 'toe-hold' to bind the 3′ end of the mRNA, PAP I generates a binding site for these exoribonucleases by adding a poly(A) tail to the 3′ end of the mRNA. Thus, cycles of polyadenylation and exoribonucleolytic attack contribute towards the correct degradation of the mRNA after the initial cleavage.

The *hok*/*sok* system mediates plasmid R1 stabilization by killing plasmid-free cells. Sok antisense RNA inhibits the translation of the *hok* mRNA, a toxic protein mRNA (Gerdes *et al.*, 1990). As Sok RNA is highly unstable, the pool of free Sok RNA decays rapidly in plasmid-free cells. The decay of Sok RNA leads to Hok protein synthesis and killing of the plasmid-free cells (Dam Mikkelsen & Gerdes, 1997). Like in the other antisense RNAs described previously, the initial step of Sok RNA decay is performed by RNase E in the single-stranded 5′ end. RNase E cleavage products are rapidly degraded from their 3′ ends by PNPase using a PAP I-dependent mechanism. Sok RNA, as well as CopA, is destabilized when RNase II is absent.

RNA degradation in eukaryotes

Because this publication has focused mainly on RNA degradation in prokaryotes, it was not the purpose of this chapter to provide a complete overview of RNA metabolism in eukaryotic cells but only pinpoints some interesting links between the systems. For a more comprehensive overview of the RNA degradation pathways in eukaryotes, readers can refer to publications focused on eukaryotes (Doma & Parker, 2007; Amaral *et al.*, 2008; Rougemaille *et al.*, 2008; Shyu *et al.*, 2008; Houseley & Tollervey, 2009; Moore & Proudfoot, 2009).

RNA degradation in eukaryotes is much more complex and involves more factors than those in prokaryotes (Houseley & Tollervey, 2009). The eukaryotic cell is divided into two main parts: the nucleus and the cytoplasm, and RNA degradation is important in both compartments. Compartmentalization causes considerable change in mRNA's fate; eukaryotic RNAs have to survive in the cell much longer than prokaryotic messengers, and the molecule synthesized in the nucleolus has to be transported to the cytoplasm for protein production. In the nucleus, aberrant transcripts are selectively degraded; RNases also act in multiple processing steps and remove the processing byproducts and a myriad of noncoding cryptic transcripts. The balance between the rate of transcription and RNA degradation regulates messenger levels. In the cytoplasm, the transcripts are translated to the proteins; therefore, in this compartment, it is very important to check the translational abilities of RNAs and remove incorrect molecules that can cause the production of aberrant proteins (Doma & Parker, 2007). In the cytoplasm, differences in the degradation rate can influence protein expression. A set of factors can affect the lifetime of the transcript including RNA-binding proteins that bind to the RNAs, and sRNAs that can drive transcripts to degradation or cause translational silencing (siRNA and miRNA) (Eulálio *et al.*, 2008; Carthew & Sontheimer, 2009).

It has been considered that in eukaryotes, the RNA degradation is mainly exonucleolytic (Fig. 3), while in prokaryotes, endonucleases have a significant impact on degradation process. In the best-studied model – yeast *S. cerevisiae* – the main enzymes involved in the degradation are exoribonucleases. Degradation in the 5′–3′ direction is performed by the Xrn1 protein in the cytoplasm and the Rat1 enzyme in the nucleus (Fritz *et al.*, 2004; Meyer *et al.*, 2004). The main yeast 3′–5′ hydrolytic exonuclease is Rrp44/(Dis3) from the RNase II family. In the nucleus, there is also another 3′–5′ exonuclease: Rrp6. Rrp44 interacts with the nine-protein ring-shapes complex to generate a ribonucleolytically active exosome, where Rrp44 is the only active RNase (Liu *et al.*, 2006b; Dziembowski *et al.*, 2007). The exosome ring is homologous to the archaeal complex with phosphorolytic nuclease activity and to the bacterial PNPase (Lorentzen *et al.*, 2007). Surprisingly, this huge protein machine lost its phosphorolytic activity in the evolution and in most eukaryotes can induce RNA degradation only when cooperating with the active component Rrp44 (Dziembowski *et al.*, 2007). Recent structural studies showed that even if the Rrp44 protein by itself is able to degrade RNA, it seems that the substrates that are delivered to this nuclease first have to pass the channel in the exosome ring structure (Bonneau *et al.*, 2009).

Research performed in the last few years proved that involvement of endonuclease activity in the RNA degradation process in eukaryotes was underestimated. Among the other examples (Huntzinger *et al.*, 2008; Eberle *et al.*, 2009), the most evident was the discovery of the endonucleolytic activity of the exosome complex; this activity is carried by the PIN domain localized in the N-terminal part of the Rrp44 protein. Rrp44, the only active component of the yeast exosome, can degrade RNA in both an exo- and an endonucleolytic manner. Because the homologues of Rrp44 from other eukaryotes also have PIN domains, it seems that endonucleolytic activity is the common feature in its RNA degradation (Lebreton *et al.*, 2008; Schaeffer *et al.*, 2009).

For a long time, the function of polyadenylation in the RNA degradation process was considered as one of the most striking differences between the eukaryotic and the prokaryotic RNA metabolism. In the eukaryotes, long poly(A) tails added by the poly(A) polymerase to the 3′ end of newly created transcripts have been considered as RNA-stabilizing elements while in the prokaryotic cell polyadenylation leads to transcript degradation. Surprisingly, it was discovered that in eukaryotes, polyadenylation can also drive RNAs to decay. The TRAMP complex composed of poly(A) polymerase, helicase and an RNA-binding protein is able to add short poly(A) tails to the aberrant transcripts, targeting them to induce rapid degradation (LaCava *et al.*, 2005). This showed that the poly(A)-dependent RNA degradation mechanism active in prokaryotes is still present in eukaryotic cells.

Last discoveries in the field of RNA degradation in eukaryotes showed that we can find much more similarities to prokaryotic systems than was previously expected. The degradation pathways in eukaryotes are obviously more complex and different in many aspects, but at the same time, many mechanisms are very similar. We can find homologues of prokaryotic enzymes that serve important functions in eukaryotic systems such as bacterial RNase II and RNase R homologue Rrp44, RNase D homologue Rrp6, the exosome ring that is structurally very similar to PNPase and others. Moreover, we can find strikingly similar mechanisms even if they are performed by factors without obvious homology. A key example is the prokaryotic antiviral defense system CRISPR, which resembles the eukaryotic RNAi mechanism (Hale *et al.*, 2009). Another example is the 5′–3′ direction exoribonucleolytic degradation pathway, which is very important in eukaryotic RNA metabolism. In the last few years, it became clear that, in spite of earlier beliefs, this pathway in prokaryotes also exists, but enzymes that are involved are not homologues of the eukaryotic ones (Mathy *et al.*, 2007). This and many other examples clearly show that evolution has led to the development of similar solutions regarding degradation mechanisms.

Eukaryotic organelles are structures of endosymbiotic prokaryotic origin; they possess their own usually reduced genome, which is expressed and transcribed, and RNAs are processed and degraded. The expression of proteins encoded in the organellar genome is, in most cases, crucial for energy management in eukaryotic cells. Many questions still remain about the RNA degradation pathways in organelles, mostly because they seem to be different in different organisms and so it is hard to find the general rules that can be applied to all systems. Nonetheless, RNA metabolic pathways in the organelles retained some characteristics of the prokaryotic ancestors. RNA degradation in chloroplasts seems to be most similar to prokaryotic process. In the higher plant genomes, we can find sequences of homologues of bacterial nucleases RNase E and RNase J that are localized in chloroplasts (Lange *et al.*, 2009). The degradation process, similar to that in bacteria, starts with endonucleolytic cleavage and is then accelerated by polyadenylation and exonucleolytic degradation by PNPase. There is also an RNase R homologue that was shown to play a role in rRNA processing (Bollenbach *et al.*, 2005).

RNA degradation pathways in the mitochondria seem to be more divergent in different organisms. Interestingly, and in contrast to the situation in chloroplasts, degradation pathways in the mitochondria are supposed to be mostly exonucleolytic. In plants, the main player seems to be PNPase, which degrades polyadenylated RNA molecules in the mitochondria (Holec *et al.*, 2006). In contrast, in yeast *S. cerevisiae*, there is no mitochondrial PNPase; instead, the main degrading machinery is the mitochondrial

degradosome complex (mtEXO), which digests RNA in the 3′–5′ direction and is composed of the homologue of RNase II-Dis3 protein and the conserved RNA DEAD-box helicase Suv3 (Dziembowski *et al.*, 2003; Malecki *et al.*, 2007). Additionally, it was suggested that there is one more potential enzyme Pet127 that can degrade RNA in the 5′–3′ direction (Fekete *et al.*, 2008). Surprisingly, there is no polyadenylaton in yeast mitochondria; instead, stabilizing functions are served by the proteins that bind to the 3′ and 5′ untranslated ends of the RNA molecules. Degradation of transcripts in human mitochondria is not well characterized. Although the data on this topic are not consistent, it seems that a homologue of bacterial PNPase is present in the mitochondria, and it was found recently that it can form a complex with the human homologue of Suv3 helicase. Suv3 is involved in RNA degradation and removal of aberrant and cryptic transcripts; the exact function of this protein is still not clear (Szczesny *et al.*, 2010). Transcripts in human mitochondria are stably polyadenylated, which, in contrast to the situation in plant mitochondria, suggests a stabilization role for poly(A) tails (Tomecki *et al.*, 2004). On the other hand, scientists also discovered polyadenylated degradation byproducts, which suggests that polyadenylation can trigger or aid transcripts' degradation; therefore, it seems that polyadenylation in human mitochondria can serve both functions (Slomovic *et al.*, 2005; Szczesny *et al.*, 2010).

Concluding remarks

Maintenance of optimal levels of RNAs at any time and under any circumstance is an extremely difficult task to achieve and requires great coordination among all the factors involved in this control. It is also assumed that there is a cross-talk between transcription and degradation to maintain the balance that is best for the survival of microorganisms. There are several examples where this is obvious, and when a specific message is more transcribed, it is also more stabilized, and vice versa.

Transcripts can have a different half-life under different growth conditions to rapidly carry out the necessary changes and adjust to adequate RNA levels. The same RNA can have a 'preferred' decay pathway, but there are examples where there are alternative degradation pathways for the same transcript, depending on which enzyme cleaves first. After cleavage, the RNA breakdown product(s) can have a distinct half-life depending on sequence and structure. Therefore, the structural characteristics of RNA stability and instability predetermine the 'fate' of an RNA, but the environment and the consequent levels and nature of the degradative enzymes will also play a determinant role in its turnover. For instance, the mRNAs expressed in heterologous systems can have a very different half-life than if they are expressed in their own microorganism. The directionality of the decay process depends on the transcript analyzed. Once we characterize the enzymes from one microorganism, we can design strategies to stabilize RNAs. Mutants have been instrumental in characterizing degradation pathways and in changing the turnover of specific transcripts, especially because a limited number of RNases intervene in the maturation and degradation of RNAs.

There are fundamental principles that govern RNA decay in all organisms. Evolution has resulted in similar functions performed by different enzymes. For instance, in *E. coli*, RNase E is one of the major endonucleases, but this enzyme is absent in *B. subtilis*. In *B. subtilis*, RNase J1 seems to take over the same function, and this enzyme is not present in *E. coli*. RNase J1 has been shown to have both endo and 5′–3′ exo activities. In yeast, 5′–3′ decay is prominent, and Rrp44/Dis3, an RNase II family enzyme, has dual endo and 3′–5′ exo activities, being an example of an optimized 'RNA degradation machine'. Sometimes, RNases also combine into complexes to speed up the decay process or confer specificity to certain targets.

It is fascinating to know that RNases themselves are strictly regulated proteins and have mechanisms to adapt them to the environment and to the levels of the other RNases. For instance, RNase R is highly increased under cold shock; the levels of PNPase and RNase II are inter-regulated and the level of RNase E is autoregulated.

Recent studies demonstrate that, between prokaryotic and eukaryotic systems, the RNA degradation mechanisms have much more similarities than expected. The mechanism of RNAi in eukaryotes has shown the power of RNA degradation mechanisms involving RNases. It is now obvious that the modulation of RNA levels and their respective proteins can be rapidly achieved. In prokaryotes, it was already known that antisense RNAs could be quite important for the control of gene expression. Moreover, the recently discovered CRISP RNAs (Karginov & Hannon, 2010), which can be considered a bacterial RNAi mechanism, have lent an extra level of complexity to the study of RNAs and bacterial RNA degradation mechanisms. It is very stimulating to work in a field of research still full of surprises! This is a thorough review, but in a few years, we are sure that there will be much more to say!

It is our hope that this review conveys some of the current excitement in research on RNA and serves as a source of inspiration for scientists entering this field.

Acknowledgements

This work was supported by grants from FCT, Portugal. We thank Clementine Dressaire for critically reading this manuscript and Miguel Luís for the graphic assistance in Fig. 2.

References

Afonyushkin T, Vecerek B, Moll I, Blasi U & Kaberdin VR (2005) Both RNase E and RNase III control the stability of *sodB* mRNA upon translational inhibition by the small regulatory RNA RyhB. *Nucleic Acids Res* **33**: 1678–1689.

Agrawal N, Dasaradhi PV, Mohmmed A, Malhotra P, Bhatnagar RK & Mukherjee SK (2003) RNA interference: biology, mechanism, and applications. *Microbiol Mol Biol R* **67**: 657–685.

Alifano P, Rivellini F, Piscitelli C, Arraiano CM, Bruni CB & Carlomagno MS (1994) Ribonuclease E provides substrates for ribonuclease P-dependent processing of a polycistronic mRNA. *Gene Dev* **8**: 3021–3031.

Altman S, Wesolowski D, Guerrier-Takada C & Li Y (2005) RNase P cleaves transient structures in some riboswitches. *P Natl Acad Sci USA* **102**: 11284–11289.

Amaral PP, Dinger ME, Mercer TR & Mattick JS (2008) The eukaryotic genome as an RNA machine. *Science* **319**: 1787–1789.

Amblar M & Arraiano CM (2005) A single mutation in *Escherichia coli* ribonuclease II inactivates the enzyme without affecting RNA binding. *FEBS J* **272**: 363–374.

Amblar M, Viegas SC, Lopez P & Arraiano CM (2004) Homologous and heterologous expression of RNase III from *Lactococcus lactis*. *Biochem Bioph Res Co* **323**: 884–890.

Amblar M, Barbas A, Fialho AM & Arraiano CM (2006) Characterization of the functional domains of *Escherichia coli* RNase II. *J Mol Biol* **360**: 921–933.

Amblar M, Barbas A, Gomez-Puertas P & Arraiano CM (2007) The role of the S1 domain in exoribonucleolytic activity: substrate specificity and multimerization. *RNA* **13**: 317–327.

Andrade JM & Arraiano CM (2008) PNPase is a key player in the regulation of small RNAs that control the expression of outer membrane proteins. *RNA* **14**: 543–551.

Andrade JM, Cairrao F & Arraiano CM (2006) RNase R affects gene expression in stationary phase: regulation of *ompA*. *Mol Microbiol* **60**: 219–228.

Andrade JM, Hajnsdorf E, Régnier P & Arraiano CM (2009a) The poly(A)-dependent degradation pathway of *rpsO* mRNA is primarily mediated by RNase R. *RNA* **15**: 316–326.

Andrade JM, Pobre V, Silva IJ, Domingues S & Arraiano CM (2009b) The role of 3′–5′ exoribonucleases in RNA degradation. *Prog Mol Biol Transl Sci* **85**: 187–229.

Apirion D & Lassar AB (1978) A conditional lethal mutant of *Escherichia coli* which affects the processing of ribosomal RNA. *J Biol Chem* **253**: 1738–1742.

Arnold TE, Yu J & Belasco JG (1998) mRNA stabilization by the *ompA* 5′ untranslated region: two protective elements hinder distinct pathways for mRNA degradation. *RNA* **4**: 319–330.

Arraiano C, Yancey SD & Kushner SR (1993) Identification of endonucleolytic cleavage sites involved in decay of *Escherichia coli trxA* mRNA. *J Bacteriol* **175**: 1043–1052.

Arraiano CM, Yancey SD & Kushner SR (1988) Stabilization of discrete mRNA breakdown products in *ams pnp rnb* multiple mutants of *Escherichia coli* K-12. *J Bacteriol* **170**: 4625–4633.

Arraiano CM, Cruz AA & Kushner SR (1997) Analysis of the *in vivo* decay of the *Escherichia coli* dicistronic *pyrF-orfF* transcript: evidence for multiple degradation pathways. *J Mol Biol* **268**: 261–272.

Arraiano CM, Barbas A & Amblar M (2008) Characterizing ribonucleases *in vitro* examples of synergies between biochemical and structural analysis. *Method Enzymol* **447**: 131–160.

Asha PK, Blouin RT, Zaniewski R & Deutscher MP (1983) Ribonuclease BN: identification and partial characterization of a new tRNA processing enzyme. *P Natl Acad Sci USA* **80**: 3301–3304.

Awano N, Inouye M & Phadtare S (2008) RNase activity of polynucleotide phosphorylase is critical at low temperature in *Escherichia coli* and is complemented by RNase II. *J Bacteriol* **190**: 5924–5933.

Awano N, Rajagopal V, Arbing M, Patel S, Hunt J, Inouye M & Phadtare S (2010) *Escherichia coli* RNase R has dual activities, helicase and RNase. *J Bacteriol* **192**: 1344–1352.

Babitzke P & Gollnick P (2001) Posttranscription initiation control of tryptophan metabolism in *Bacillus subtilis* by the *trp* RNA-binding attenuation protein (TRAP), anti-TRAP, and RNA structure. *J Bacteriol* **183**: 5795–5802.

Babitzke P & Kushner SR (1991) The Ams (altered mRNA stability) protein and ribonuclease E are encoded by the same structural gene of *Escherichia coli*. *P Natl Acad Sci USA* **88**: 1–5.

Babitzke P, Granger L, Olszewski J & Kushner SR (1993) Analysis of mRNA decay and rRNA processing in *Escherichia coli* multiple mutants carrying a deletion in RNase III. *J Bacteriol* **175**: 229–239.

Baer MF, Wesolowski D & Altman S (1989) Characterization *in vitro* of the defect in a temperature-sensitive mutant of the protein subunit of RNase P from *Escherichia coli*. *J Bacteriol* **171**: 6862–6866.

Baker KE & Mackie GA (2003) Ectopic RNase E sites promote bypass of 5′-end-dependent mRNA decay in *Escherichia coli*. *Mol Microbiol* **47**: 75–88.

Barbas A, Andrade JM, Fialho AM & Arraiano CM (2006) Ribonucleases e controlo pós-transcripcional da expressão génica. *O Mundo do RNA – Novos Desafios e Perspectivas Futuras* (Arraiano CM & Fialho AM, eds), pp. 119–139. Lidel Edições Técnicas, Lisbon, Portugal.

Barbas A, Matos RG, Amblar M, Lopez-Vinas E, Gomez-Puertas P & Arraiano CM (2008) New insights into the mechanism of RNA degradation by ribonuclease II: identification of the residue responsible for setting the RNase II end-product. *J Biol Chem* **283**: 13070–13076.

Barbas A, Matos RG, Amblar M, Lopez-Vinas E, Gomez-Puertas P & Arraiano CM (2009) Determination of key residues for catalysis and RNA cleavage specificity: one mutation turns RNase II into a 'SUPER-ENZYME'. *J Biol Chem* **284**: 20486–20498.

Bardwell JC, Régnier P, Chen SM, Nakamura Y, Grunberg-Manago M & Court DL (1989) Autoregulation of RNase III operon by mRNA processing. *EMBO J* **8**: 3401–3407.

Barnett TC, Bugrysheva JV & Scott JR (2007) Role of mRNA stability in growth phase regulation of gene expression in the group A *Streptococcus*. *J Bacteriol* **189**: 1866–1873.

Bechhofer DH (2009) Messenger RNA decay and maturation in *Bacillus subtilis*. *Prog Mol Biol Transl Sci* **85**: 231–273.

Belitsky BR, Gustafsson MC, Sonenshein AL & Von Wachenfeldt C (1997) An lrp-like gene of *Bacillus subtilis* involved in branched-chain amino acid transport. *J Bacteriol* **179**: 5448–5457.

Beran RK & Simons RW (2001) Cold-temperature induction of *Escherichia coli* polynucleotide phosphorylase occurs by reversal of its autoregulation. *Mol Microbiol* **39**: 112–125.

Bermúdez-Cruz RM, Fernandez-Ramírez F, Kameyama-Kawabe L & Montañez C (2005) Conserved domains in polynucleotide phosphorylase among eubacteria. *Biochimie* **87**: 737–745.

Bernstein JA, Lin PH, Cohen SN & Lin-Chao S (2004) Global analysis of *Escherichia coli* RNA degradosome function using DNA microarrays. *P Natl Acad Sci USA* **101**: 2758–2763.

Binnie U, Wong K, McAteer S & Masters M (1999) Absence of RNase III alters the pathway by which RNAI, the antisense inhibitor of ColE1 replication, decays. *Microbiology* **145**: 3089–3100.

Blaszczyk J, Tropea JE, Bubunenko M, Routzahn KM, Waugh DS, Court DL & Ji X (2001) Crystallographic and modeling studies of RNase III suggest a mechanism for double-stranded RNA cleavage. *Structure* **9**: 1225–1236.

Blaszczyk J, Gan J, Tropea JE, Court DL, Waugh DS & Ji X (2004) Noncatalytic assembly of ribonuclease III with double-stranded RNA. *Structure* **12**: 457–466.

Bollenbach TJ, Lange H, Gutierrez R, Erhardt M, Stern DB & Gagliardi D (2005) RNR1, a 3′–5′ exoribonuclease belonging to the RNR superfamily, catalyzes 3′ maturation of chloroplast ribosomal RNAs in *Arabidopsis thaliana*. *Nucleic Acids Res* **33**: 2751–2763.

Bonneau F, Basquin J, Ebert J, Lorentzen E & Conti E (2009) The yeast exosome functions as a macromolecular cage to channel RNA substrates for degradation. *Cell* **139**: 547–559.

Bothwell AL, Garber RL & Altman S (1976) Nucleotide sequence and *in vitro* processing of a precursor molecule to *Escherichia coli* 4.5 S RNA. *J Biol Chem* **251**: 7709–7716.

Bralley P & Jones GH (2003) Overexpression of the polynucleotide phosphorylase gene (pnp) of *Streptomyces antibioticus* affects mRNA stability and poly(A) tail length but not ppGpp levels. *Microbiology* **149**: 2173–2182.

Bralley P, Gust B, Chang S, Chater KF & Jones GH (2006) RNA 3′-tail synthesis in *Streptomyces*: *in vitro* and *in vivo* activities of RNase PH, the SCO3896 gene product and polynucleotide phosphorylase. *Microbiology* **152**: 627–636.

Braun F, Hajnsdorf E & Régnier P (1996) Polynucleotide phosphorylase is required for the rapid degradation of the RNase E-processed *rps*O mRNA of *Escherichia coli* devoid of its 3′ hairpin. *Mol Microbiol* **19**: 997–1005.

Briani F, Del Favero M, Capizzuto R *et al.* (2007) Genetic analysis of polynucleotide phosphorylase structure and functions. *Biochimie* **89**: 145–157.

Briani F, Curti S, Rossi F, Carzaniga T, Mauri P & Dehò G (2008) Polynucleotide phosphorylase hinders mRNA degradation upon ribosomal protein S1 overexpression in *Escherichia coli*. *RNA* **14**: 2417–2429.

Britton RA, Wen T, Schaefer L *et al.* (2007) Maturation of the 5′ end of *Bacillus subtilis* 16S rRNA by the essential ribonuclease YkqC/RNase J1. *Mol Microbiol* **63**: 127–138.

Brown JW & Reeve JN (1986) Polyadenylated RNA isolated from the archaebacterium *Halobacterium halobium*. *J Bacteriol* **166**: 686–688.

Brown S & Coleman G (1975) Messenger ribonucleic acid content of *Bacillus amyloliquefaciens* throughout its growth cycle compared with *Bacillus subtilis* 168. *J Mol Biol* **96**: 345–352.

Bugrysheva JV & Scott JR (2009) The ribonucleases J1 and J2 are essential for growth and have independent roles in mRNA decay in *Streptococcus pyogenes*. *Mol Microbiol* **75**: 731–743.

Burgin AB, Parodos K, Lane DJ & Pace NR (1990) The excision of intervening sequences from *Salmonella* 23S ribosomal RNA. *Cell* **60**: 405–414.

Butland G, Peregrin-Alvarez JM, Li J *et al.* (2005) Interaction network containing conserved and essential protein complexes in *Escherichia coli*. *Nature* **433**: 531–537.

Buttner K, Wenig K & Hopfner KP (2005) Structural framework for the mechanism of archaeal exosomes in RNA processing. *Mol Cell* **20**: 461–471.

Cairrão F & Arraiano CM (2006) The role of endoribonucleases in the regulation of RNase R. *Biochem Bioph Res Co* **343**: 731–737.

Cairrão F, Chora A, Zilhão R, Carpousis AJ & Arraiano CM (2001) RNase II levels change according to the growth conditions: characterization of *gmr*, a new *Escherichia coli* gene involved in the modulation of RNase II. *Mol Microbiol* **39**: 1550–1561.

Cairrão F, Cruz A, Mori H & Arraiano CM (2003) Cold shock induction of RNase R and its role in the maturation of the quality control mediator SsrA/tmRNA. *Mol Microbiol* **50**: 1349–1360.

Calin-Jageman I & Nicholson AW (2003) RNA structure-dependent uncoupling of substrate recognition and cleavage by *Escherichia coli* ribonuclease III. *Nucleic Acids Res* **31**: 2381–2392.

Callaghan AJ, Aurikko JP, Ilag LL *et al.* (2004) Studies of the RNA degradosome-organizing domain of the *Escherichia coli* ribonuclease RNase E. *J Mol Biol* **340**: 965–979.

Callaghan AJ, Marcaida MJ, Stead JA, McDowall KJ, Scott WG & Luisi BF (2005a) Structure of *Escherichia coli* RNase E catalytic domain and implications for RNA turnover. *Nature* **437**: 1187–1191.

Callaghan AJ, Redko Y, Murphy LM *et al.* (2005b) 'Zn-link': a metal-sharing interface that organizes the quaternary structure and catalytic site of the endoribonuclease, RNase E. *Biochemistry* **44**: 4667–4675.

Campos-Guillen J, Arvizu-Gomez JL, Jones GH & Olmedo-Alvarez G (2010) Characterization of tRNACys processing in a conditional *Bacillus subtilis* CCase mutant reveals the

participation of RNase R in its quality control. *Microbiology* **156**: 2102–2111.
Cannistraro VJ & Kennell D (1989) Purification and characterization of ribonuclease M and mRNA degradation in *Escherichia coli*. *Eur J Biochem* **181**: 363–370.
Cannistraro VJ & Kennell D (1991) RNase I*, a form of RNase I, and mRNA degradation in *Escherichia coli*. *J Bacteriol* **173**: 4653–4659.
Cannistraro VJ & Kennell D (1994) The processive reaction mechanism of ribonuclease II. *J Mol Biol* **243**: 930–943.
Cannistraro VJ & Kennell D (1999) The reaction mechanism of ribonuclease II and its interaction with nucleic acid secondary structures. *Biochim Biophys Acta* **1433**: 170–187.
Cardenas PP, Carrasco B, Sanchez H, Deikus G, Bechhofer DH & Alonso JC (2009) *Bacillus subtilis* polynucleotide phosphorylase 3′-to-5′ DNase activity is involved in DNA repair. *Nucleic Acids Res* **37**: 4157–4169.
Carpousis AJ, Vanzo NF & Raynal LC (1999) mRNA degradation. A tale of poly(A) and multiprotein machines. *Trends Genet* **15**: 24–28.
Carpousis AJ, Luisi BF & McDowall KJ (2009) Endonucleolytic initiation of mRNA decay in *Escherichia coli*. *Prog Mol Biol Transl Sci* **85**: 91–135.
Carthew RW & Sontheimer EJ (2009) Origins and mechanisms of miRNAs and siRNAs. *Cell* **136**: 642–655.
Carzaniga T, Briani F, Zangrossi S, Merlino G, Marchi P & Dehò G (2009) Autogenous regulation of *Escherichia coli* polynucleotide phosphorylase expression revisited. *J Bacteriol* **191**: 1738–1748.
Celesnik H, Deana A & Belasco JG (2007) Initiation of RNA decay in *Escherichia coli* by 5′ pyrophosphate removal. *Mol Cell* **27**: 79–90.
Cerritelli SM & Crouch RJ (2009) Ribonuclease H: the enzymes in eukaryotes. *FEBS J* **276**: 1494–1505.
Chandran V & Luisi BF (2006) Recognition of enolase in the *Escherichia coli* RNA degradosome. *J Mol Biol* **358**: 8–15.
Chandran V, Poljak L, Vanzo NF *et al.* (2007) Recognition and cooperation between the ATP-dependent RNA helicase RhlB and ribonuclease RNase E. *J Mol Biol* **367**: 113–132.
Charpentier X, Faucher SP, Kalachikov S & Shuman HA (2008) Loss of RNase R induces competence development in *Legionella pneumophila*. *J Bacteriol* **190**: 8126–8136.
Chen JL, Nolan JM, Harris ME & Pace NR (1998) Comparative photocross-linking analysis of the tertiary structures of *Escherichia coli* and *Bacillus subtilis* RNase P RNAs. *EMBO J* **17**: 1515–1525.
Cheng ZF & Deutscher MP (2002) Purification and characterization of the *Escherichia coli* exoribonuclease RNase R. Comparison with RNase II. *J Biol Chem* **277**: 21624–21629.
Cheng ZF & Deutscher MP (2003) Quality control of ribosomal RNA mediated by polynucleotide phosphorylase and RNase R. *P Natl Acad Sci USA* **100**: 6388–6393.
Cheng ZF & Deutscher MP (2005) An important role for RNase R in mRNA decay. *Mol Cell* **17**: 313–318.
Cheng ZF, Zuo Y, Li Z, Rudd KE & Deutscher MP (1998) The *vacB* gene required for virulence in *Shigella flexneri* and *Escherichia coli* encodes the exoribonuclease RNase R. *J Biol Chem* **273**: 14077–14080.
Choi JM, Park EY, Kim JH, Chang SK & Cho Y (2004) Probing the functional importance of the hexameric ring structure of RNase PH. *J Biol Chem* **279**: 755–764.
Choonee N, Even S, Zig L & Putzer H (2007) Ribosomal protein L20 controls expression of the *Bacillus subtilis infC* operon via a transcription attenuation mechanism. *Nucleic Acids Res* **35**: 1578–1588.
Chung DH, Min Z, Wang BC & Kushner SR (2010) Single amino acid changes in the predicted RNase H domain of *Escherichia coli* RNase G lead to complementation of RNase E deletion mutants. *RNA* **16**: 1371–1385.
Clements MO, Eriksson S, Thompson A, Lucchini S, Hinton JC, Normark S & Rhen M (2002) Polynucleotide phosphorylase is a global regulator of virulence and persistency in *Salmonella enterica*. *P Natl Acad Sci USA* **99**: 8784–8789.
Coburn GA & Mackie GA (1996a) Overexpression, purification, and properties of *Escherichia coli* ribonuclease II. *J Biol Chem* **271**: 1048–1053.
Coburn GA & Mackie GA (1996b) Differential sensitivities of portions of the mRNA for ribosomal protein S20 to 3′-exonucleases dependent on oligoadenylation and RNA secondary structure. *J Biol Chem* **271**: 15776–15781.
Coburn GA & Mackie GA (1998) Reconstitution of the degradation of the mRNA for ribosomal protein S20 with purified enzymes. *J Mol Biol* **279**: 1061–1074.
Coburn GA, Miao X, Briant DJ & Mackie GA (1999) Reconstitution of a minimal RNA degradosome demonstrates functional coordination between a 3′ exonuclease and a DEAD-box RNA helicase. *Gene Dev* **13**: 2594–2603.
Commichau FM, Rothe FM, Herzberg C *et al.* (2009) Novel activities of glycolytic enzymes in *Bacillus subtilis*: interactions with essential proteins involved in mRNA processing. *Mol Cell Proteomics* **8**: 1350–1360.
Condon C & Putzer H (2002) The phylogenetic distribution of bacterial ribonucleases. *Nucleic Acids Res* **30**: 5339–5346.
Condon C, Brechemier-Baey D, Beltchev B, Grunberg-Manago M & Putzer H (2001) Identification of the gene encoding the 5S ribosomal RNA maturase in *Bacillus subtilis*: mature 5S rRNA is dispensable for ribosome function. *RNA* **7**: 242–253.
Condon C, Rourera J, Brechemier-Baey D & Putzer H (2002) Ribonuclease M5 has few, if any, mRNA substrates in *Bacillus subtilis*. *J Bacteriol* **184**: 2845–2849.
Court DL (1993) RNA processing and degradation by RNase III. *Control of Messenger RNA Stability* (Belasco GJ & Brawerman G, ed), pp. 71–116. Academic Press, New York.
Cudny H & Deutscher MP (1980) Apparent involvement of ribonuclease D in the 3′ processing of tRNA precursors. *P Natl Acad Sci USA* **77**: 837–841.
Cudny H, Zaniewski R & Deutscher MP (1981) *Escherichia coli* RNase D. Catalytic properties and substrate specificity. *J Biol Chem* **256**: 5633–5637.

Czerwoniec A, Dunin-Horkawicz S, Purta E *et al.* (2009) MODOMICS: a database of RNA modification pathways. 2008 update. *Nucleic Acids Res* **37**: D118–D121.

Dahlberg AE, Dahlberg JE, Lund E *et al.* (1978) Processing of the 5′ end of *Escherichia coli* 16S ribosomal RNA. *P Natl Acad Sci USA* **75**: 3598–3602.

Dam Mikkelsen N & Gerdes K (1997) Sok antisense RNA from plasmid R1 is functionally inactivated by RNase E and polyadenylated by poly(A) polymerase I. *Mol Microbiol* **26**: 311–320.

Dasgupta S, Fernandez L, Kameyama L, Inada T, Nakamura Y, Pappas A & Court DL (1998) Genetic uncoupling of the dsRNA-binding and RNA cleavage activities of the *Escherichia coli* endoribonuclease RNase III – the effect of dsRNA binding on gene expression. *Mol Microbiol* **28**: 629–640.

Datta AK & Niyogi K (1975) A novel oligoribonuclease of *Escherichia coli*. II. Mechanism of action. *J Biol Chem* **250**: 7313–7319.

Davies JF II, Hostomska Z, Hostomsky Z, Jordan SR & Matthews DA (1991) Crystal structure of the ribonuclease H domain of HIV-1 reverse transcriptase. *Science* **252**: 88–95.

Deana A, Celesnik H & Belasco JG (2008) The bacterial enzyme RppH triggers messenger RNA degradation by 5′ pyrophosphate removal. *Nature* **451**: 355–358.

Deikus G & Bechhofer DH (2009) *Bacillus subtilis trp* Leader RNA: RNase J1 endonuclease cleavage specificity and PNPase processing. *J Biol Chem* **284**: 26394–26401.

Deikus G, Babitzke P & Bechhofer DH (2004) Recycling of a regulatory protein by degradation of the RNA to which it binds. *P Natl Acad Sci USA* **101**: 2747–2751.

Deikus G, Condon C & Bechhofer DH (2008) Role of *Bacillus subtilis* RNase J1 endonuclease and 5′-exonuclease activities in *trp* leader RNA turnover. *J Biol Chem* **283**: 17158–17167.

de la Sierra-Gallay IL, Pellegrini O & Condon C (2005) Structural basis for substrate binding, cleavage and allostery in the tRNA maturase RNase Z. *Nature* **433**: 657–661.

de la Sierra-Gallay IL, Zig L, Jamalli A & Putzer H (2008) Structural insights into the dual activity of RNase J. *Nat Struct Mol Biol* **15**: 206–212.

Del Favero M, Mazzantini E, Briani F, Zangrossi S, Tortora P & Dehò G (2008) Regulation of *Escherichia coli* polynucleotide phosphorylase by ATP. *J Biol Chem* **283**: 27355–27359.

Deutscher MP (1990) Ribonucleases, tRNA nucleotidyltransferase, and the 3′ processing of tRNA. *Prog Nucleic Acid Re* **39**: 209–240.

Deutscher MP (2006) Degradation of RNA in bacteria: comparison of mRNA and stable RNA. *Nucleic Acids Res* **34**: 659–666.

Deutscher MP (2009) Maturation and degradation of ribosomal RNA in bacteria. *Prog Mol Biol Transl Sci* **85**: 369–391.

Deutscher MP & Marlor CW (1985) Purification and characterization of *Escherichia coli* RNase T. *J Biol Chem* **260**: 7067–7071.

Deutscher MP & Reuven NB (1991) Enzymatic basis for hydrolytic versus phosphorolytic mRNA degradation in *Escherichia coli* and *Bacillus subtilis*. *P Natl Acad Sci USA* **88**: 3277–3280.

Deutscher MP, Marlor CW & Zaniewski R (1985) RNase T is responsible for the end-turnover of tRNA in *Escherichia coli*. *P Natl Acad Sci USA* **82**: 6427–6430.

Deutscher MP, Marshall GT & Cudny H (1988) RNase PH: an *Escherichia coli* phosphate-dependent nuclease distinct from polynucleotide phosphorylase. *P Natl Acad Sci USA* **85**: 4710–4714.

Dittmar KA, Mobley EM, Radek AJ & Pan T (2004) Exploring the regulation of tRNA distribution on the genomic scale. *J Mol Biol* **337**: 31–47.

Diwa A, Bricker AL, Jain C & Belasco JG (2000) An evolutionarily conserved RNA stem-loop functions as a sensor that directs feedback regulation of RNase E gene expression. *Gene Dev* **14**: 1249–1260.

Doma MK & Parker R (2007) RNA quality control in eukaryotes. *Cell* **131**: 660–668.

Domingues S, Matos RG, Reis FP, Fialho AM, Barbas A & Arraiano CM (2009) Biochemical characterization of the RNase II family of exoribonucleases from the human pathogens *Salmonella typhimurium* and *Streptococcus pneumoniae*. *Biochemistry* **48**: 11848–11857.

Donovan WP & Kushner SR (1983) Cloning and physical analysis of the *pyr*F gene (coding for orotidine-5′-phosphate decarboxylase) from *Escherichia coli* K-12. *Gene* **25**: 39–48.

Donovan WP & Kushner SR (1986) Polynucleotide phosphorylase and ribonuclease II are required for cell viability and mRNA turnover in *Escherichia coli* K-12. *P Natl Acad Sci USA* **83**: 120–124.

Drider D & Condon C (2004) The continuing story of endoribonuclease III. *J Mol Microb Biotech* **8**: 195–200.

Drider D, Santos JM, Arraiano CM & Lopez P (1998) RNA processing is involved in the post-transcriptional control of the citQRP operon from *Lactococcus lactis* biovar *diacetylactis*. *Mol Gen Genet* **258**: 9–15.

Drider D, Garcia-Quintans N, Santos JM, Arraiano CM & Lopez P (1999) A comparative analysis of the citrate permease P mRNA stability in *Lactococcus lactis* biovar diacetylactis and *Escherichia coli*. *FEMS Microbiol Lett* **172**: 115–122.

Dulebohn DP, Cho HJ & Karzai AW (2006) Role of conserved surface amino acids in binding of SmpB protein to SsrA RNA. *J Biol Chem* **281**: 28536–28545.

Dutta T & Deutscher MP (2009) Catalytic properties of RNase BN/RNase Z from *Escherichia coli*: RNase BN is both an exo- and endoribonuclease. *J Biol Chem* **284**: 15425–15431.

Dutta T & Deutscher MP (2010) Mode of action of RNase BN/RNase Z on tRNA precursors: RNase BN does not remove the CCA sequence from tRNA. *J Biol Chem*, in press.

Dziembowski A, Piwowarski J, Hoser R *et al.* (2003) The yeast mitochondrial degradosome. Its composition, interplay between RNA helicase and RNase activities and the role in mitochondrial RNA metabolism. *J Biol Chem* **278**: 1603–1611.

Dziembowski A, Lorentzen E, Conti E & Seraphin B (2007) A single subunit, Dis3, is essentially responsible for yeast exosome core activity. *Nat Struct Mol Biol* **14**: 15–22.

Eberle AB, Lykke-Andersen S, Muhlemann O & Jensen TH (2009) SMG6 promotes endonucleolytic cleavage of nonsense mRNA in human cells. *Nat Struct Mol Biol* **16**: 49–55.

Erova TE, Kosykh VG, Fadl AA, Sha J, Horneman AJ & Chopra AK (2008) Cold shock exoribonuclease R (VacB) is involved in *Aeromonas hydrophila* pathogenesis. *J Bacteriol* **190**: 3467–3474.

Eulálio A, Huntzinger E & Izaurralde E (2008) Getting to the root of miRNA-mediated gene silencing. *Cell* **132**: 9–14.

Evans D, Marquez SM & Pace NR (2006) RNase P: interface of the RNA and protein worlds. *Trends Biochem Sci* **31**: 333–341.

Even S, Pellegrini O, Zig L, Labas V, Vinh J, Brechemmier-Baey D & Putzer H (2005) Ribonucleases J1 and J2: two novel endoribonucleases in *B. subtilis* with functional homology to *E. coli* RNase E. *Nucleic Acids Res* **33**: 2141–2152.

Evguenieva-Hackenberg E & Klug G (2000) RNase III processing of intervening sequences found in helix 9 of 23S rRNA in the alpha subclass of Proteobacteria. *J Bacteriol* **182**: 4719–4729.

Evguenieva-Hackenberg E & Klug G (2009) RNA degradation in Archaea and Gram-negative bacteria different from *Escherichia coli*. *Prog Mol Biol Transl Sci* **85**: 275–317.

Evguenieva-Hackenberg E, Schiltz E & Klug G (2002) Dehydrogenases from all three domains of life cleave RNA. *J Biol Chem* **277**: 46145–46150.

Evguenieva-Hackenberg E, Walter P, Hochleitner E, Lottspeich F & Klug G (2003) An exosome-like complex in *Sulfolobus solfataricus*. *EMBO Rep* **4**: 889–893.

Evguenieva-Hackenberg E, Roppelt V, Finsterseifer P & Klug G (2008) Rrp4 and Csl4 are needed for efficient degradation but not for polyadenylation of synthetic and natural RNA by the archaeal exosome. *Biochemistry* **47**: 13158–13168.

Ezraty B, Dahlgren B & Deutscher MP (2005) The RNase Z homologue encoded by *Escherichia coli elaC* gene is RNase BN. *J Biol Chem* **280**: 16542–16545.

Falaleeva MV, Chetverina HV, Ugarov VI, Uzlova EA & Chetverin AB (2008) Factors influencing RNA degradation by *Thermus thermophilus* polynucleotide phosphorylase. *FEBS J* **275**: 2214–2226.

Fang M, Zeisberg WM, Condon C, Ogryzko V, Danchin A & Mechold U (2009) Degradation of nanoRNA is performed by multiple redundant RNases in *Bacillus subtilis*. *Nucleic Acids Res* **37**: 5114–5125.

Farhoud MH, Wessels HJ, Steenbakkers PJ *et al.* (2005) Protein complexes in the archaeon *Methanothermobacter thermautotrophicus* analyzed by blue native/SDS-PAGE and mass spectrometry. *Mol Cell Proteomics* **4**: 1653–1663.

Fekete Z, Ellis TP, Schonauer MS & Dieckmann CL (2008) Pet127 governs a $5' \rightarrow 3'$-exonuclease important in maturation of apocytochrome b mRNA in *Saccharomyces cerevisiae*. *J Biol Chem* **283**: 3767–3772.

Fiedler TJ, Vincent HA, Zuo Y, Gavrialov O & Malhotra A (2004) Purification and crystallization of *Escherichia coli* oligoribonuclease. *Acta Crystallogr D* **60**: 736–739.

Folichon M, Arluison V, Pellegrini O, Huntzinger E, Régnier P & Hajnsdorf E (2003) The poly(A) binding protein Hfq protects RNA from RNase E and exoribonucleolytic degradation. *Nucleic Acids Res* **31**: 7302–7310.

Folichon M, Marujo PE, Arluison V, Le Derout J, Pellegrini O, Hajnsdorf E & Régnier P (2005) Fate of mRNA extremities generated by intrinsic termination: detailed analysis of reactions catalyzed by ribonuclease II and poly(A) polymerase. *Biochimie* **87**: 819–826.

Fonseca P, Moreno R & Rojo F (2008) Genomic analysis of the role of RNase R in the turnover of *Pseudomonas putida* mRNAs. *J Bacteriol* **190**: 6258–6263.

Fournier MJ & Ozeki H (1985) Structure and organization of the transfer ribonucleic acid genes of *Escherichia coli* K-12. *Microbiol Rev* **49**: 379–397.

Frazão C, McVey CE, Amblar M, Barbas A, Vonrhein C, Arraiano CM & Carrondo MA (2006) Unravelling the dynamics of RNA degradation by ribonuclease II and its RNA-bound complex. *Nature* **443**: 110–114.

Freire P, Amaral JD, Santos JM & Arraiano CM (2006) Adaptation to carbon starvation: RNase III ensures normal expression levels of bolA1p mRNA and sigma(S). *Biochimie* **88**: 341–346.

Fritz DT, Bergman N, Kilpatrick WJ, Wilusz CJ & Wilusz J (2004) Messenger RNA decay in mammalian cells: the exonuclease perspective. *Cell Biochem Biophys* **41**: 265–278.

Fusi P, Tedeschi G, Aliverti A, Ronchi S, Tortora P & Guerritore A (1993) Ribonucleases from the extreme thermophilic archaebacterium *S. solfataricus*. *Eur J Biochem* **211**: 305–310.

Gan J, Tropea JE, Austin BP, Court DL, Waugh DS & Ji X (2006) Structural insight into the mechanism of double-stranded RNA processing by ribonuclease III. *Cell* **124**: 355–366.

Gao J, Lee K, Zhao M *et al.* (2006) Differential modulation of *E. coli* mRNA abundance by inhibitory proteins that alter the composition of the degradosome. *Mol Microbiol* **61**: 394–406.

García-Mena J, Das A, Sánchez-Trujillo A, Portier C & Montanez C (1999) A novel mutation in the KH domain of polynucleotide phosphorylase affects autoregulation and mRNA decay in *Escherichia coli*. *Mol Microbiol* **33**: 235–248.

Geer LY, Domrachev M, Lipman DJ & Bryant SH (2002) CDART: protein homology by domain architecture. *Genome Res* **12**: 1619–1623.

Gegenheimer P & Apirion D (1975) *Escherichia coli* ribosomal ribonucleic acids are not cut from an intact precursor molecule. *J Biol Chem* **250**: 2407–2409.

Gerdes K, Thisted T & Martinussen J (1990) Mechanism of post-segregational killing by the *hok/sok* system of plasmid R1: *sok* antisense RNA regulates formation of a *hok* mRNA species correlated with killing of plasmid-free cells. *Mol Microbiol* **4**: 1807–1818.

Ghora BK & Apirion D (1978) Structural analysis and *in vitro* processing to p5 rRNA of a 9S RNA molecule isolated from an *rne* mutant of *E. coli*. *Cell* **15**: 1055–1066.

Ghosh S & Deutscher MP (1999) Oligoribonuclease is an essential component of the mRNA decay pathway. *P Natl Acad Sci USA* **96**: 4372–4377.

Gimple O & Schon A (2001) *In vitro* and *in vivo* processing of cyanelle tmRNA by RNase P. *Biol Chem* **382**: 1421–1429.

Givskov M & Molin S (1984) Copy mutants of plasmid R1: effects of base pair substitutions in the *cop*A gene on the replication control system. *Mol Gen Genet* **194**: 286–292.

Gobert A, Gutmann B, Taschner A *et al.* (2010) A single *Arabidopsis* organellar protein has RNase P activity. *Nat Struct Mol Biol* **17**: 740–744.

Godefroy T (1970) Kinetics of polymerization and phosphorolysis reactions of *Escherichia coli* polynucleotide phosphorylase. Evidence for multiple binding of polynucleotide in phosphorolysis. *Eur J Biochem* **14**: 222–231.

Grossman D & van Hoof A (2006) RNase II structure completes group portrait of 3′ exoribonucleases. *Nat Struct Mol Biol* **13**: 760–761.

Guillier M, Gottesman S & Storz G (2006) Modulating the outer membrane with small RNAs. *Gene Dev* **20**: 2338–2348.

Haddad N, Burns CM, Bolla JM, Prévost H, Federighi M, Drider D & Cappelier JM (2009) Long-term survival of *Campylobacter jejuni* at low temperatures is dependent on polynucleotide phosphorylase activity. *Appl Environ Microb* **75**: 7310–7318.

Hahn J, Luttinger A & Dubnau D (1996) Regulatory inputs for the synthesis of ComK, the competence transcription factor of *Bacillus subtilis*. *Mol Microbiol* **21**: 763–775.

Hajnsdorf E, Steier O, Coscoy L, Teysset L & Régnier P (1994) Roles of RNase E, RNase II and PNPase in the degradation of the *rpsO* transcripts of *Escherichia coli*: stabilizing function of RNase II and evidence for efficient degradation in an *ams pnp rnb* mutant. *EMBO J* **13**: 3368–3377.

Hajnsdorf E, Braun F, Haugel-Nielsen J & Régnier P (1995) Polyadenylylation destabilizes the *rps*O mRNA of *Escherichia coli*. *P Natl Acad Sci USA* **92**: 3973–3977.

Hale CR, Zhao P, Olson S *et al.* (2009) RNA-guided RNA cleavage by a CRISPR RNA–Cas protein complex. *Cell* **139**: 945–956.

Hall TA & Brown JW (2002) Archaeal RNase P has multiple protein subunits homologous to eukaryotic nuclear RNase P proteins. *RNA* **8**: 296–306.

Hankins JS, Zappavigna C, Prud'homme-Genereux A & Mackie GA (2007) Role of RNA structure and susceptibility to RNase E in regulation of a cold shock mRNA, cspA mRNA. *J Bacteriol* **189**: 4353–4358.

Harlow LS, Kadziola A, Jensen KF & Larsen S (2004) Crystal structure of the phosphorolytic exoribonuclease RNase PH from *Bacillus subtilis* and implications for its quaternary structure and tRNA binding. *Protein Sci* **13**: 668–677.

Hartmann RK, Heinrich J, Schlegl J & Schuster H (1995) Precursor of C4 antisense RNA of bacteriophages P1 and P7 is a substrate for RNase P of *Escherichia coli*. *P Natl Acad Sci USA* **92**: 5822–5826.

Hartmann RK, Gossringer M, Spath B, Fischer S & Marchfelder A (2009) The making of tRNAs and more – RNase P and tRNase Z. *Prog Mol Biol Transl Sci* **85**: 319–368.

Hasenohrl D, Lombo T, Kaberdin V, Londei P & Blasi U (2008) Translation initiation factor a/eIF2(-gamma) counteracts 5′ to 3′ mRNA decay in the archaeon *Sulfolobus solfataricus*. *P Natl Acad Sci USA* **105**: 2146–2150.

Hayes CS & Keiler KC (2010) Beyond ribosome rescue: tmRNA and co-translational processes. *FEBS Lett* **584**: 413–419.

Hayes F & Vasseur M (1976) Processing of the 17-S *Escherichia coli* precursor RNA in the 27-S pre-ribosomal particle. *Eur J Biochem* **61**: 433–442.

Henkin TM (2008) Riboswitch RNAs: using RNA to sense cellular metabolism. *Gene Dev* **22**: 3383–3390.

Henkin TM & Yanofsky C (2002) Regulation by transcription attenuation in bacteria: how RNA provides instructions for transcription termination/antitermination decisions. *Bioessays* **24**: 700–707.

Herskovitz MA & Bechhofer DH (2000) Endoribonuclease RNase III is essential in *Bacillus subtilis*. *Mol Microbiol* **38**: 1027–1033.

Higgins CF, McLaren RS & Newbury SF (1988) Repetitive extragenic palindromic sequences, mRNA stability and gene expression: evolution by gene conversion? A review. *Gene* **72**: 3–14.

Holec S, Lange H, Kuhn K, Alioua M, Borner T & Gagliardi D (2006) Relaxed transcription in *Arabidopsis* mitochondria is counterbalanced by RNA stability control mediated by polyadenylation and polynucleotide phosphorylase. *Mol Cell Biol* **26**: 2869–2876.

Holzmann J, Frank P, Loffler E, Bennett KL, Gerner C & Rossmanith W (2008) RNase P without RNA: identification and functional reconstitution of the human mitochondrial tRNA processing enzyme. *Cell* **135**: 462–474.

Hong SJ, Tran QA & Keiler KC (2005) Cell cycle-regulated degradation of tmRNA is controlled by RNase R and SmpB. *Mol Microbiol* **57**: 565–575.

Hopper AK, Pai DA & Engelke DR (2010) Cellular dynamics of tRNAs and their genes. *FEBS Lett* **584**: 310–317.

Hou YM & Perona JJ (2010) Stereochemical mechanisms of tRNA methyltransferases. *FEBS Lett* **584**: 278–286.

Houseley J & Tollervey D (2009) The many pathways of RNA degradation. *Cell* **136**: 763–776.

Huntzinger E, Boisset S, Saveanu C *et al.* (2005) *Staphylococcus aureus* RNAIII and the endoribonuclease III coordinately regulate *spa* gene expression. *EMBO J* **24**: 824–835.

Huntzinger E, Kashima I, Fauser M, Sauliere J & Izaurralde E (2008) SMG6 is the catalytic endonuclease that cleaves mRNAs containing nonsense codons in metazoan. *RNA* **14**: 2609–2617.

Hutchison CA, Peterson SN, Gill SR *et al.* (1999) Global transposon mutagenesis and a minimal *Mycoplasma* genome. *Science* **286**: 2165–2169.

Inokuchi H & Yamao F (1995) Structure and expression of prokaryotic tRNA genes. *tRNA: Structure, Biosynthesis, and Function* (Söll D & RajBhandary UL, eds), pp. 17–30. ASM Press, Washington, DC.

Irie M (1997) RNase T1/RNase T2 family RNases. *Ribonucleases: Structure and Functions* (D'Allessio GRJ, ed), pp. 101–124. Academic Press, San Diego, CA.

Ishii R, Nureki O & Yokoyama S (2003) Crystal structure of the tRNA processing enzyme RNase PH from *Aquifex aeolicus*. *J Biol Chem* **278**: 32397–32404.

Ishii R, Minagawa A, Takaku H, Takagi M, Nashimoto M & Yokoyama S (2005) Crystal structure of the tRNA 3′ processing endoribonuclease tRNase Z from *Thermotoga maritima*. *J Biol Chem* **280**: 14138–14144.

Itaya M, Omori A, Kanaya S, Crouch RJ, Tanaka T & Kondo K (1999) Isolation of RNase H genes that are essential for growth of *Bacillus subtilis* 168. *J Bacteriol* **181**: 2118–2123.

Jagannath A & Wood M (2007) RNA interference based gene therapy for neurological disease. *Brief Funct Genomic Proteomic* **6**: 40–49.

Jäger S, Fuhrmann O, Heck C, Hebermehl M, Schiltz E, Rauhut R & Klug G (2001) An mRNA degrading complex in *Rhodobacter capsulatus*. *Nucleic Acids Res* **29**: 4581–4588.

Jäger S, Evguenieva-Hackenberg E & Klug G (2004) Temperature-dependent processing of the *cspA* mRNA in *Rhodobacter capsulatus*. *Microbiology* **150**: 687–695.

Jain C & Belasco JG (1995) Autoregulation of RNase E synthesis in *Escherichia coli*. *Nucleic Acids Symp Ser* **33**: 85–88.

Janssen BD & Hayes CS (2009) Kinetics of paused ribosome recycling in *Escherichia coli*. *J Mol Biol* **394**: 251–267.

Jarrige A, Brechemier-Baey D, Mathy N, Duche O & Portier C (2002) Mutational analysis of polynucleotide phosphorylase from *Escherichia coli*. *J Mol Biol* **321**: 397–409.

Jarrige AC, Mathy N & Portier C (2001) PNPase autocontrols its expression by degrading a double-stranded structure in the *pnp* mRNA leader. *EMBO J* **20**: 6845–6855.

Jaskiewicz L & Filipowicz W (2008) Role of Dicer in posttranscriptional RNA silencing. *Curr Top Microbiol* **320**: 77–97.

Jensen KF, Larsen JN, Schack L & Sivertsen A (1984) Studies on the structure and expression of *Escherichia coli pyr*C, *pyr*D, and *pyr*F using the cloned genes. *Eur J Biochem* **140**: 343–352.

Jensen KF, Andersen JT & Poulsen P (1992) Overexpression and rapid purification of the *orfE*/*rph* gene product, RNase PH of *Escherichia coli*. *J Biol Chem* **267**: 17147–17152.

Jiang X & Belasco JG (2004) Catalytic activation of multimeric RNase E and RNase G by 5′-monophosphorylated RNA. *P Natl Acad Sci USA* **101**: 9211–9216.

Jiang X, Diwa A & Belasco JG (2000) Regions of RNase E important for 5′-end-dependent RNA cleavage and autoregulated synthesis. *J Bacteriol* **182**: 2468–2475.

Jinek M & Doudna JA (2009) A three-dimensional view of the molecular machinery of RNA interference. *Nature* **457**: 405–412.

Johansen J, Rasmussen AA, Overgaard M & Valentin-Hansen P (2006) Conserved small non-coding RNAs that belong to the sigmaE regulon: role in down-regulation of outer membrane proteins. *J Mol Biol* **364**: 1–8.

Kaberdin VR & Blasi U (2006) Translation initiation and the fate of bacterial mRNAs. *FEMS Microbiol Rev* **30**: 967–979.

Kaberdin VR, Miczak A, Jakobsen JS, Lin-Chao S, McDowall KJ & von Gabain A (1998) The endoribonucleolytic N-terminal half of *Escherichia coli* RNase E is evolutionarily conserved in *Synechocystis* sp. and other bacteria but not the C-terminal half, which is sufficient for degradosome assembly. *P Natl Acad Sci USA* **95**: 11637–11642.

Kane JF (1995) Effects of rare codon clusters on high-level expression of heterologous proteins in *Escherichia coli*. *Curr Opin Biotech* **6**: 494–500.

Karginov FV & Hannon GJ (2010) The CRISPR system: small RNA-guided defense in bacteria and archaea. *Mol Cell* **37**: 7–19.

Karzai AW, Susskind MM & Sauer RT (1999) SmpB, a unique RNA-binding protein essential for the peptide-tagging activity of SsrA (tmRNA). *EMBO J* **18**: 3793–3799.

Kelly KO & Deutscher MP (1992) Characterization of *Escherichia coli* RNase PH. *J Biol Chem* **267**: 17153–17158.

Kelly KO, Reuven NB, Li Z & Deutscher MP (1992) RNase PH is essential for tRNA processing and viability in RNase-deficient *Escherichia coli* cells. *J Biol Chem* **267**: 16015–16018.

Khemici V & Carpousis AJ (2004) The RNA degradosome and poly(A) polymerase of *Escherichia coli* are required *in vivo* for the degradation of small mRNA decay intermediates containing REP-stabilizers. *Mol Microbiol* **51**: 777–790.

Khemici V, Toesca I, Poljak L, Vanzo NF & Carpousis AJ (2004) The RNase E of *Escherichia coli* has at least two binding sites for DEAD-box RNA helicases: functional replacement of RhlB by RhlE. *Mol Microbiol* **54**: 1422–1430.

Khemici V, Poljak L, Luisi BF & Carpousis AJ (2008) The RNase E of *Escherichia coli* is a membrane-binding protein. *Mol Microbiol* **70**: 799–813.

Kime L, Jourdan SS, Stead JA, Hidalgo-Sastre A & McDowall KJ (2009) Rapid cleavage of RNA by RNase E in the absence of 5′-monophosphate stimulation. *Mol Microbiol* **76**: 590–604.

Kirsebom LA & Trobro S (2009) RNase P RNA-mediated cleavage. *IUBMB Life* **61**: 189–200.

Kirsebom LA, Baer MF & Altman S (1988) Differential effects of mutations in the protein and RNA moieties of RNase P on the efficiency of suppression by various tRNA suppressors. *J Mol Biol* **204**: 879–888.

Kitamura S, Fujishima K, Sato A, Tsuchiya D, Tomita M & Kanai A (2010) Characterization of RNase HII substrate recognition using RNase HII-Argonaute chimeric enzymes from *Pyrococcus furiosus*. *Biochem J* **426**: 337–344.

Ko JH & Altman S (2007) OLE RNA, an RNA motif that is highly conserved in several extremophilic bacteria, is a substrate for and can be regulated by RNase P RNA. *P Natl Acad Sci USA* **104**: 7815–7820.

Ko JH, Han K, Kim Y *et al.* (2008) Dual function of RNase E for control of M1 RNA biosynthesis in *Escherichia coli*. *Biochemistry* **47**: 762–770.

Koonin EV, Wolf YI & Aravind L (2001) Prediction of the archaeal exosome and its connections with the proteasome and the translation and transcription machineries by a comparative-genomic approach. *Genome Res* **11**: 240–252.

Koslover DJ, Callaghan AJ, Marcaida MJ, Garman EF, Martick M, Scott WG & Luisi BF (2008) The crystal structure of the *Escherichia coli* RNase E apoprotein and a mechanism for RNA degradation. *Structure* **16**: 1238–1244.

Kulms D, Schafer G & Hahn U (1995) SaRD, a new protein isolated from the extremophile archaeon *Sulfolobus acidocaldarius*, is a thermostable ribonuclease with DNA-binding properties. *Biochem Bioph Res Co* **214**: 646–652.

LaCava J, Houseley J, Saveanu C, Petfalski E, Thompson E, Jacquier A & Tollervey D (2005) RNA degradation by the exosome is promoted by a nuclear polyadenylation complex. *Cell* **121**: 713–724.

Lalonde MS, Zuo Y, Zhang J, Gong X, Wu S, Malhotra A & Li Z (2007) Exoribonuclease R in *Mycoplasma genitalium* can carry out both RNA processing and degradative functions and is sensitive to RNA ribose methylation. *RNA* **13**: 1957–1968.

Lamontagne B, Larose S, Boulanger J & Elela SA (2001) The RNase III family: a conserved structure and expanding functions in eukaryotic dsRNA metabolism. *Curr Issues Mol Biol* **3**: 71–78.

Lange H, Sement FM, Canaday J & Gagliardi D (2009) Polyadenylation-assisted RNA degradation processes in plants. *Trends Plant Sci* **14**: 497–504.

Lebreton A, Tomecki R, Dziembowski A & Seraphin B (2008) Endonucleolytic RNA cleavage by a eukaryotic exosome. *Nature* **456**: 993–996.

Le Derout J, Boni IV, Régnier P & Hajnsdorf E (2010) Hfq affects mRNA levels independently of degradation. *BMC Mol Biol* **11**: 17.

Lee K & Cohen SN (2003) A *Streptomyces coelicolor* functional orthologue of *Escherichia coli* RNase E shows shuffling of catalytic and PNPase-binding domains. *Mol Microbiol* **48**: 349–360.

Lee K, Bernstein JA & Cohen SN (2002) RNase G complementation of *rne* null mutation identifies functional interrelationships with RNase E in *Escherichia coli*. *Mol Microbiol* **43**: 1445–1456.

Lee K, Zhan X, Gao J *et al.* (2003) RraA. A protein inhibitor of RNase E activity that globally modulates RNA abundance in *E. coli*. *Cell* **114**: 623–634.

Li H & Nicholson AW (1996) Defining the enzyme binding domain of a ribonuclease III processing signal. Ethylation interference and hydroxyl radical footprinting using catalytically inactive RNase III mutants. *EMBO J* **15**: 1421–1433.

Li Y & Altman S (2003) A specific endoribonuclease, RNase P, affects gene expression of polycistronic operon mRNAs. *P Natl Acad Sci USA* **100**: 13213–13218.

Li Z & Deutscher MP (1995) The tRNA processing enzyme RNase T is essential for maturation of 5S RNA. *P Natl Acad Sci USA* **92**: 6883–6886.

Li Z & Deutscher MP (1996) Maturation pathways for *E. coli* tRNA precursors: a random multienzyme process *in vivo*. *Cell* **86**: 503–512.

Li Z & Deutscher MP (2002) RNase E plays an essential role in the maturation of *Escherichia coli* tRNA precursors. *RNA* **8**: 97–109.

Li Z, Zhan L & Deutscher MP (1996) *Escherichia coli* RNase T functions *in vivo* as a dimer dependent on cysteine 168. *J Biol Chem* **271**: 1133–1137.

Li Z, Pandit S & Deutscher MP (1998) 3′ Exoribonucleolytic trimming is a common feature of the maturation of small, stable RNAs in *Escherichia coli*. *P Natl Acad Sci USA* **95**: 2856–2861.

Li Z, Pandit S & Deutscher MP (1999a) Maturation of 23S ribosomal RNA requires the exoribonuclease RNase T. *RNA* **5**: 139–146.

Li Z, Pandit S & Deutscher MP (1999b) RNase G (CafA protein) and RNase E are both required for the 5′ maturation of 16S ribosomal RNA. *EMBO J* **18**: 2878–2885.

Li Z, Reimers S, Pandit S & Deutscher MP (2002) RNA quality control: degradation of defective transfer RNA. *EMBO J* **21**: 1132–1138.

Lin-Chao S & Cohen SN (1991) The rate of processing and degradation of antisense RNAI regulates the replication of ColE1-type plasmids *in vivo*. *Cell* **65**: 1233–1242.

Lin-Chao S, Wei CL & Lin YT (1999) RNase E is required for the maturation of SsrA RNA and normal SsrA RNA peptide-tagging activity. *P Natl Acad Sci USA* **96**: 12406–12411.

Lin PH & Lin-Chao S (2005) RhlB helicase rather than enolase is the beta-subunit of the *Escherichia coli* polynucleotide phosphorylase (PNPase)-exoribonucleolytic complex. *P Natl Acad Sci USA* **102**: 16590–16595.

Liou GG, Jane WN, Cohen SN, Lin NS & Lin-Chao S (2001) RNA degradosomes exist *in vivo* in *Escherichia coli* as multicomponent complexes associated with the cytoplasmic membrane via the N-terminal region of ribonuclease E. *P Natl Acad Sci USA* **98**: 63–68.

Liou GG, Chang HY, Lin CS & Lin-Chao S (2002) DEAD box RhlB RNA helicase physically associates with exoribonuclease PNPase to degrade double-stranded RNA independent of the degradosome-assembling region of RNase E. *J Biol Chem* **277**: 41157–41162.

Littauer YZ & Soreq H (1982) *Polynucleotide Phosphorylase*. Academic Press, New York.

Liu F & Altman S (1994) Differential evolution of substrates for an RNA enzyme in the presence and absence of its protein cofactor. *Cell* **77**: 1093–1100.

Liu F & Altman S (2009) *Ribonuclease P*. Springer-Verlag, New York.

Liu H & Kiledjian M (2006a) Decapping the message: a beginning or an end. *Biochem Soc T* **34**: 35–38.

Liu Q, Greimann JC & Lima CD (2006b) Reconstitution, activities, and structure of the eukaryotic RNA exosome. *Cell* **127**: 1223–1237.

Lombo TB & Kaberdin VR (2008) RNA processing in *Aquifex aeolicus* involves RNase E/G and an RNase P-like activity. *Biochem Bioph Res Co* **366**: 457–463.

Lopez PJ, Marchand I, Joyce SA & Dreyfus M (1999) The C-terminal half of RNase E, which organizes the *Escherichia coli* degradosome, participates in mRNA degradation but not rRNA processing *in vivo*. *Mol Microbiol* **33**: 188–199.

Lorentzen E, Walter P, Fribourg S, Evguenieva-Hackenberg E, Klug G & Conti E (2005) The archaeal exosome core is a hexameric ring structure with three catalytic subunits. *Nat Struct Mol Biol* **12**: 575–581.

Lorentzen E, Dziembowski A, Lindner D, Seraphin B & Conti E (2007) RNA channelling by the archaeal exosome. *EMBO Rep* **8**: 470–476.

Lu C, Ding F & Ke A (2010) Crystal structure of the *S. solfataricus* archaeal exosome reveals conformational flexibility in the RNA-binding ring. *PLoS One* **5**: e8739.

Lundberg U & Altman S (1995) Processing of the precursor to the catalytic RNA subunit of RNase P from *Escherichia coli*. *RNA* **1**: 327–334.

Luttinger A, Hahn J & Dubnau D (1996) Polynucleotide phosphorylase is necessary for competence development in *Bacillus subtilis*. *Mol Microbiol* **19**: 343–356.

Ma Y, Chan CY & He ML (2007) RNA interference and antiviral therapy. *World J Gastroentero* **13**: 5169–5179.

Mackie GA (1991) Specific endonucleolytic cleavage of the mRNA for ribosomal protein S20 of *Escherichia coli* requires the product of the *ams* gene *in vivo* and *in vitro*. *J Bacteriol* **173**: 2488–2497.

Mackie GA (1992) Secondary structure of the mRNA for ribosomal protein S20. Implications for cleavage by ribonuclease E. *J Biol Chem* **267**: 1054–1061.

Mackie GA (1998) Ribonuclease E is a 5′-end-dependent endonuclease. *Nature* **395**: 720–723.

Mackie GA (2000) Stabilization of circular rpsT mRNA demonstrates the 5′-end dependence of RNase E action *in vivo*. *J Biol Chem* **275**: 25069–25072.

Mackie GA & Parsons GD (1983) Tandem promoters in the gene for ribosomal protein S20. *J Biol Chem* **258**: 7840–7846.

MacRae IJ & Doudna JA (2007) Ribonuclease revisited: structural insights into ribonuclease III family enzymes. *Curr Opin Struc Biol* **17**: 138–145.

Mader U, Zig L, Kretschmer J, Homuth G & Putzer H (2008) mRNA processing by RNases J1 and J2 affects *Bacillus subtilis* gene expression on a global scale. *Mol Microbiol* **70**: 183–196.

Malecki M, Jedrzejczak R, Stepien PP & Golik P (2007) *In vitro* reconstitution and characterization of the yeast mitochondrial degradosome complex unravels tight functional interdependence. *J Mol Biol* **372**: 23–36.

Marcaida MJ, DePristo MA, Chandran V, Carpousis AJ & Luisi BF (2006) The RNA degradosome: life in the fast lane of adaptive molecular evolution. *Trends Biochem Sci* **31**: 359–365.

Marck C & Grosjean H (2002) tRNomics: analysis of tRNA genes from 50 genomes of Eukarya, Archaea, and Bacteria reveals anticodon-sparing strategies and domain-specific features. *RNA* **8**: 1189–1232.

Marck C & Grosjean H (2003) Identification of BHB splicing motifs in intron-containing tRNAs from 18 archaea: evolutionary implications. *RNA* **9**: 1516–1531.

Marszalkowski M, Willkomm DK & Hartmann RK (2008) 5′-End maturation of tRNA in *Aquifex aeolicus*. *Biol Chem* **389**: 395–403.

Marujo PE, Hajnsdorf E, Le Derout J, Andrade R, Arraiano CM & Régnier P (2000) RNase II removes the oligo(A) tails that destabilize the *rps*O mRNA of *Escherichia coli*. *RNA* **6**: 1185–1193.

Marujo PE, Braun F, Haugel-Nielsen J, Le Derout J, Arraiano CM & Régnier P (2003) Inactivation of the decay pathway initiated at an internal site by RNase E promotes poly(A)-dependent degradation of the *rps*O mRNA in *Escherichia coli*. *Mol Microbiol* **50**: 1283–1294.

Massire C, Jaeger L & Westhof E (1998) Derivation of the three-dimensional architecture of bacterial ribonuclease P RNAs from comparative sequence analysis. *J Mol Biol* **279**: 773–793.

Mathy N, Jarrige AC, Robert-Le Meur M & Portier C (2001) Increased expression of *Escherichia coli* polynucleotide phosphorylase at low temperatures is linked to a decrease in the efficiency of autocontrol. *J Bacteriol* **183**: 3848–3854.

Mathy N, Benard L, Pellegrini O, Daou R, Wen T & Condon C (2007) 5′-to-3′ exoribonuclease activity in bacteria: role of RNase J1 in rRNA maturation and 5′ stability of mRNA. *Cell* **129**: 681–692.

Mathy N, Hebert A, Mervelet P *et al.* (2010) *Bacillus subtilis* ribonucleases J1 and J2 form a complex with altered enzyme behaviour. *Mol Microbiol* **75**: 489–498.

Matos RG, Barbas A & Arraiano CM (2009) RNase R mutants elucidate the catalysis of structured RNA: RNA-binding domains select the RNAs targeted for degradation. *Biochem J* **423**: 291–301.

Matsunaga J, Simons EL & Simons RW (1996) RNase III autoregulation: structure and function of *rncO*, the posttranscriptional 'operator'. *RNA* **2**: 1228–1240.

Matus-Ortega ME, Regonesi ME, Pina-Escobedo A, Tortora P, Dehò G & García-Mena J (2007) The KH and S1 domains of *Escherichia coli* polynucleotide phosphorylase are necessary for autoregulation and growth at low temperature. *Biochim Biophys Acta* **1769**: 194–203.

McDowall KJ, Hernandez RG, Lin-Chao S & Cohen SN (1993) The *ams-1* and *rne-3071* temperature-sensitive mutations in the *ams* gene are in close proximity to each other and cause substitutions within a domain that resembles a product of the *Escherichia coli mre* locus. *J Bacteriol* **175**: 4245–4249.

McDowall KJ, Kaberdin VR, Wu SW, Cohen SN & Lin-Chao S (1995) Site-specific RNase E cleavage of oligonucleotides and inhibition by stem-loops. *Nature* **374**: 287–290.

Mechold U, Ogryzko V, Ngo S & Danchin A (2006) Oligoribonuclease is a common downstream target of lithium-

induced pAp accumulation in *Escherichia coli* and human cells. *Nucleic Acids Res* **34**: 2364–2373.

Mechold U, Fang G, Ngo S, Ogryzko V & Danchin A (2007) YtqI from *Bacillus subtilis* has both oligoribonuclease and pAp-phosphatase activity. *Nucleic Acids Res* **35**: 4552–4561.

Melefors O & von Gabain A (1988) Site-specific endonucleolytic cleavages and the regulation of stability of *E. coli omp*A mRNA. *Cell* **52**: 893–901.

Melefors O & von Gabain A (1991) Genetic studies of cleavage-initiated mRNA decay and processing of ribosomal 9S RNA show that the *Escherichia coli ams* and *rne* loci are the same. *Mol Microbiol* **5**: 857–864.

Meng W & Nicholson AW (2008) Heterodimer-based analysis of subunit and domain contributions to double-stranded RNA processing by *Escherichia coli* RNase III *in vitro*. *Biochem J* **410**: 39–48.

Meyer S, Temme C & Wahle E (2004) Messenger RNA turnover in eukaryotes: pathways and enzymes. *Crit Rev Biochem Mol* **39**: 197–216.

Mian IS (1997) Comparative sequence analysis of ribonucleases HII, III, II PH and D. *Nucleic Acids Res* **25**: 3187–3195.

Miczak A, Kaberdin VR, Wei CL & Lin-Chao S (1996) Proteins associated with RNase E in a multicomponent ribonucleolytic complex. *P Natl Acad Sci USA* **93**: 3865–3869.

Minagawa A, Takaku H, Takagi M & Nashimoto M (2004) A novel endonucleolytic mechanism to generate the CCA 3′ termini of tRNA molecules in *Thermotoga maritima*. *J Biol Chem* **279**: 15688–15697.

Misra TK & Apirion D (1979) RNase E, an RNA processing enzyme from *Escherichia coli*. *J Biol Chem* **254**: 11154–11159.

Mitchell P, Petfalski E, Shevchenko A, Mann M & Tollervey D (1997) The exosome: a conserved eukaryotic RNA processing complex containing multiple 3′ → 5′ exoribonucleases. *Cell* **91**: 457–466.

Mitra S & Bechhofer DH (1994) Substrate specificity of an RNase III-like activity from *Bacillus subtilis*. *J Biol Chem* **269**: 31450–31456.

Mohanty BK & Kushner SR (1999) Analysis of the function of *Escherichia coli* poly(A) polymerase I in RNA metabolism. *Mol Microbiol* **34**: 1094–1108.

Mohanty BK & Kushner SR (2000a) Polynucleotide phosphorylase, RNase II and RNase E play different roles in the *in vivo* modulation of polyadenylation in *Escherichia coli*. *Mol Microbiol* **36**: 982–994.

Mohanty BK & Kushner SR (2000b) Polynucleotide phosphorylase functions both as a 3′ right-arrow 5′ exonuclease and a poly(A) polymerase in *Escherichia coli*. *P Natl Acad Sci USA* **97**: 11966–11971.

Mohanty BK & Kushner SR (2002) Polyadenylation of *Escherichia coli* transcripts plays an integral role in regulating intracellular levels of polynucleotide phosphorylase and RNase E. *Mol Microbiol* **45**: 1315–1324.

Mohanty BK & Kushner SR (2003) Genomic analysis in *Escherichia coli* demonstrates differential roles for polynucleotide phosphorylase and RNase II in mRNA abundance and decay. *Mol Microbiol* **50**: 645–658.

Mohanty BK & Kushner SR (2006) The majority of *Escherichia coli* mRNAs undergo post-transcriptional modification in exponentially growing cells. *Nucleic Acids Res* **34**: 5695–5704.

Mohanty BK & Kushner SR (2010) Processing of the *Escherichia coli leuX* tRNA transcript, encoding tRNALeu5, requires either the 3′ → 5′ exoribonuclease polynucleotide phosphorylase or RNase P to remove the Rho-independent transcription terminator. *Nucleic Acids Res* **38**: 597–607.

Mohanty BK, Maples VF & Kushner SR (2004) The Sm-like protein Hfq regulates polyadenylation dependent mRNA decay in *Escherichia coli*. *Mol Microbiol* **54**: 905–920.

Moore MJ & Proudfoot NJ (2009) Pre-mRNA processing reaches back to transcription and ahead to translation. *Cell* **136**: 688–700.

Morita T, Maki K & Aiba H (2005) RNase E-based ribonucleoprotein complexes: mechanical basis of mRNA destabilization mediated by bacterial noncoding RNAs. *Gene Dev* **19**: 2176–2186.

Mudd EA & Higgins CF (1993) *Escherichia coli* endoribonuclease RNase E: autoregulation of expression and site-specific cleavage of mRNA. *Mol Microbiol* **9**: 557–568.

Mudd EA, Krisch HM & Higgins CF (1990) RNase E, an endoribonuclease, has a general role in the chemical decay of *Escherichia coli* mRNA: evidence that *rne* and *ams* are the same genetic locus. *Mol Microbiol* **4**: 2127–2135.

Nakamura A, Koide Y, Miyazaki H, Kitamura A, Masaki H, Beppu T & Uozumi T (1992) Gene cloning and characterization of a novel extracellular ribonuclease of *Bacillus subtilis*. *Eur J Biochem* **209**: 121–127.

Nashimoto M (1997) Distribution of both lengths and 5′ terminal nucleotides of mammalian pre-tRNA 3′ trailers reflects properties of 3′ processing endoribonuclease. *Nucleic Acids Res* **25**: 1148–1154.

Neubauer C, Gao Y-G, Andersen KR *et al.* (2009) The structural basis for mRNA recognition and cleavage by the ribosome-dependent endonuclease RelE. *Cell* **139**: 1084–1095.

Newbury SF, Smith NH & Higgins CF (1987) Differential mRNA stability controls relative gene expression within a polycistronic operon. *Cell* **51**: 1131–1143.

Nicholson AW (1999) Function, mechanism and regulation of bacterial ribonucleases. *FEMS Microbiol Rev* **23**: 371–390.

Nilsson G, Belasco JG, Cohen SN & von Gabain A (1984) Growth-rate dependent regulation of mRNA stability in *Escherichia coli*. *Nature* **312**: 75–77.

Nishio SY & Itoh T (2008) The effects of RNA degradation enzymes on antisense RNAI controlling ColE2 plasmid copy number. *Plasmid* **60**: 174–180.

Niyogi SK & Datta AK (1975) A novel oligoribonuclease of *Escherichia coli*. Isolation and properties. *J Biol Chem* **250**: 7307–7312.

Noirot-Gros MF, Dervyn E, Wu LJ, Mervelet P, Errington J, Ehrlich SD & Noirot P (2002) An expanded view of bacterial DNA replication. *P Natl Acad Sci USA* **99**: 8342–8347.

Nowotny M & Yang W (2006) Stepwise analyses of metal ions in RNase H catalysis from substrate destabilization to product release. *EMBO J* **25**: 1924–1933.

Nurmohamed S, Vaidialingam B, Callaghan AJ & Luisi BF (2009) Crystal structure of *Escherichia coli* polynucleotide phosphorylase core bound to RNase E, RNA and manganese: implications for catalytic mechanism and RNA degradosome assembly. *J Mol Biol* **389**: 17–33.

O'Hara EB, Chekanova JA, Ingle CA, Kushner ZR, Peters E & Kushner SR (1995) Polyadenylylation helps regulate mRNA decay in *Escherichia coli*. *P Natl Acad Sci USA* **92**: 1807–1811.

Oguro A, Kakeshita H, Nakamura K, Yamane K, Wang W & Bechhofer DH (1998) *Bacillus subtilis* RNase III cleaves both 5′- and 3′-sites of the small cytoplasmic RNA precursor. *J Biol Chem* **273**: 19542–19547.

Ohnishi Y, Nishiyama Y, Sato R, Kameyama S & Horinouchi S (2000) An oligoribonuclease gene in *Streptomyces griseus*. *J Bacteriol* **182**: 4647–4653.

Ohtani N, Haruki M, Morikawa M, Crouch RJ, Itaya M & Kanaya S (1999) Identification of the genes encoding Mn^{2+}-dependent RNase HII and Mg^{2+}-dependent RNase HIII from *Bacillus subtilis*: classification of RNases H into three families. *Biochemistry* **38**: 605–618.

Okada Y, Wachi M, Hirata A, Suzuki K, Nagai K & Matsuhashi M (1994) Cytoplasmic axial filaments in *Escherichia coli* cells: possible function in the mechanism of chromosome segregation and cell division. *J Bacteriol* **176**: 917–922.

Olmedo G & Guzman P (2008) Mini-III, a fourth class of RNase III catalyses maturation of the *Bacillus subtilis* 23S ribosomal RNA. *Mol Microbiol* **68**: 1073–1076.

Ono M & Kuwano M (1979) A conditional lethal mutation in an *Escherichia coli* strain with a longer chemical lifetime of messenger RNA. *J Mol Biol* **129**: 343–357.

Ono M & Kuwano M (1980) Chromosomal location of a gene for chemical longevity of messenger ribonucleic acid in a temperature-sensitive mutant of *Escherichia coli*. *J Bacteriol* **142**: 325–326.

Oppenheim AB, Kornitzer D, Altuvia S & Court DL (1993) Posttranscriptional control of the lysogenic pathway in bacteriophage lambda. *Prog Nucleic Acid Re* **46**: 37–49.

Ost KA & Deutscher MP (1991) *Escherichia coli orfE* (upstream of *pyrE*) encodes RNase PH. *J Bacteriol* **173**: 5589–5591.

Otsuka Y & Yonesaki T (2005) A novel endoribonuclease, RNase LS, in *Escherichia coli*. *Genetics* **169**: 13–20.

Oussenko IA, Sanchez R & Bechhofer DH (2002) *Bacillus subtilis* YhaM, a member of a new family of 3′-to-5′ exonucleases in gram-positive bacteria. *J Bacteriol* **184**: 6250–6259.

Oussenko IA, Abe T, Ujiie H, Muto A & Bechhofer DH (2005) Participation of 3′-to-5′ exoribonucleases in the turnover of *Bacillus subtilis* mRNA. *J Bacteriol* **187**: 2758–2767.

Ow MC & Kushner SR (2002) Initiation of tRNA maturation by RNase E is essential for cell viability in *E. coli*. *Gene Dev* **16**: 1102–1115.

Ow MC, Liu Q & Kushner SR (2000) Analysis of mRNA decay and rRNA processing in *Escherichia coli* in the absence of RNase E-based degradosome assembly. *Mol Microbiol* **38**: 854–866.

Ow MC, Liu Q, Mohanty BK, Andrew ME, Maples VF & Kushner SR (2002) RNase E levels in *Escherichia coli* are controlled by a complex regulatory system that involves transcription of the *rne* gene from three promoters. *Mol Microbiol* **43**: 159–171.

Ow MC, Perwez T & Kushner SR (2003) RNase G of *Escherichia coli* exhibits only limited functional overlap with its essential homologue, RNase E. *Mol Microbiol* **49**: 607–622.

Palanisamy SK, Fletcher C, Tanjung L, Katz ME & Cheetham BF (2009) Deletion of the C-terminus of polynucleotide phosphorylase increases twitching motility, a virulence characteristic of the anaerobic bacterial pathogen *Dichelobacter nodosus*. *FEMS Microbiol Lett* **302**: 39–45.

Peck-Miller KA & Altman S (1991) Kinetics of the processing of the precursor to 4.5 S RNA, a naturally occurring substrate for RNase P from *Escherichia coli*. *J Mol Biol* **221**: 1–5.

Pedersen K, Zavialov AV, Pavlov MY, Elf J, Gerdes K & Ehrenberg M (2003) The bacterial toxin RelE displays codon-specific cleavage of mRNAs in the ribosomal A site. *Cell* **112**: 131–140.

Pellegrini O, Nezzar J, Marchfelder A, Putzer H & Condon C (2003) Endonucleolytic processing of CCA-less tRNA precursors by RNase Z in *Bacillus subtilis*. *EMBO J* **22**: 4534–4543.

Pepe CM, Maslesa-Galic S & Simons RW (1994) Decay of the IS10 antisense RNA by 3′ exoribonucleases: evidence that RNase II stabilizes RNA-OUT against PNPase attack. *Mol Microbiol* **13**: 1133–1142.

Pertzev AV & Nicholson AW (2006b) Characterization of RNA sequence determinants and antideterminants of processing reactivity for a minimal substrate of *Escherichia coli* ribonuclease III. *Nucleic Acids Res* **34**: 3708–3721.

Perwez T & Kushner SR (2006a) RNase Z in *Escherichia coli* plays a significant role in mRNA decay. *Mol Microbiol* **60**: 723–737.

Phizicky EM & Alfonzo JD (2010) Do all modifications benefit all tRNAs? *FEBS Lett* **584**: 265–271.

Piazza F, Zappone M, Sana M, Briani F & Dehò G (1996) Polynucleotide phosphorylase of *Escherichia coli* is required for the establishment of bacteriophage P4 immunity. *J Bacteriol* **178**: 5513–5521.

Portier C & Regnier P (1984) Expression of the *rpsO* and *pnp* genes: structural analysis of a DNA fragment carrying their control regions. *Nucleic Acids Res* **12**: 6091–6102.

Portier C, Dondon L, Grunberg-Manago M & Régnier P (1987) The first step in the functional inactivation of the *Escherichia coli* polynucleotide phosphorylase messenger is a ribonuclease III processing at the 5′ end. *EMBO J* **6**: 2165–2170.

Portnoy V & Schuster G (2006) RNA polyadenylation and degradation in different Archaea; roles of the exosome and RNase R. *Nucleic Acids Res* **34**: 5923–5931.

Portnoy V, Evguenieva-Hackenberg E, Klein F, Walter P, Lorentzen E, Klug G & Schuster G (2005) RNA polyadenylation in Archaea: not observed in *Haloferax* while the exosome polynucleotidylates RNA in *Sulfolobus*. *EMBO Rep* **6**: 1188–1193.

Prud'homme-Genereux A, Beran RK, Iost I, Ramey CS, Mackie GA & Simons RW (2004) Physical and functional interactions among RNase E, polynucleotide phosphorylase and the cold-shock protein, CsdA: evidence for a 'cold shock degradosome'. *Mol Microbiol* **54**: 1409–1421.

Pruijn GJ (2005) Doughnuts dealing with RNA. *Nat Struct Mol Biol* **12**: 562–564.

Purusharth RI, Klein F, Sulthana S *et al.* (2005) Exoribonuclease R interacts with endoribonuclease E and an RNA helicase in the psychrotrophic bacterium *Pseudomonas syringae* Lz4W. *J Biol Chem* **280**: 14572–14578.

Purusharth RI, Madhuri B & Ray MK (2007) Exoribonuclease R in *Pseudomonas syringae* is essential for growth at low temperature and plays a novel role in the 3′ end processing of 16 and 5 S ribosomal RNA. *J Biol Chem* **282**: 16267–16277.

Py B, Causton H, Mudd EA & Higgins CF (1994) A protein complex mediating mRNA degradation in *Escherichia coli*. *Mol Microbiol* **14**: 717–729.

Py B, Higgins CF, Krisch HM & Carpousis AJ (1996) A DEAD-box RNA helicase in the *Escherichia coli* RNA degradosome. *Nature* **381**: 169–172.

Randau L, Schroder I & Soll D (2008) Life without RNase P. *Nature* **453**: 120–123.

Rasmussen AA, Eriksen M, Gilany K, Udesen C, Franch T, Petersen C & Valentin-Hansen P (2005) Regulation of *omp*A mRNA stability: the role of a small regulatory RNA in growth phase-dependent control. *Mol Microbiol* **58**: 1421–1429.

Redko Y, Bechhofer DH & Condon C (2008) Mini-III, an unusual member of the RNase III family of enzymes, catalyses 23S ribosomal RNA maturation in *B. subtilis*. *Mol Microbiol* **68**: 1096–1106.

Régnier P & Grunberg-Manago M (1990) RNase III cleavages in non-coding leaders of *Escherichia coli* transcripts control mRNA stability and genetic expression. *Biochimie* **72**: 825–834.

Régnier P & Portier C (1986) Initiation, attenuation and RNase III processing of transcripts from the *Escherichia coli* operon encoding ribosomal protein S15 and polynucleotide phosphorylase. *J Mol Biol* **187**: 23–32.

Regonesi ME, Briani F, Ghetta A, Zangrossi S, Ghisotti D, Tortora P & Deho G (2004) A mutation in polynucleotide phosphorylase from *Escherichia coli* impairing RNA binding and degradosome stability. *Nucleic Acids Res* **32**: 1006–1017.

Regonesi ME, Del Favero M, Basilico F *et al.* (2006) Analysis of the *Escherichia coli* RNA degradosome composition by a proteomic approach. *Biochimie* **88**: 151–161.

Reva ON, Weinel C, Weinel M, Bohm K, Stjepandic D, Hoheisel JD & Tummler B (2006) Functional genomics of stress response in *Pseudomonas putida* KT2440. *J Bacteriol* **188**: 4079–4092.

Robert-Le Meur M & Portier C (1992) *E.coli* polynucleotide phosphorylase expression is autoregulated through an RNase III-dependent mechanism. *EMBO J* **11**: 2633–2641.

Robert-Le Meur M & Portier C (1994) Polynucleotide phosphorylase of *Escherichia coli* induces the degradation of its RNase III processed messenger by preventing its translation. *Nucleic Acids Res* **22**: 397–403.

Robertson HD, Webster RE & Zinder ND (1968) Purification and properties of ribonuclease III from *Escherichia coli*. *J Biol Chem* **243**: 82–91.

Robertson HD, Altman S & Smith JD (1972) Purification and properties of a specific *Escherichia coli* ribonuclease which cleaves a tyrosine transfer ribonucleic acid precursor. *J Biol Chem* **247**: 5243–5251.

Rosenzweig JA, Weltman G, Plano GV & Schesser K (2005) Modulation of *Yersinia* type three secretion system by the S1 domain of polynucleotide phosphorylase. *J Biol Chem* **280**: 156–163.

Rosenzweig JA, Chromy B, Echeverry A *et al.* (2007) Polynucleotide phosphorylase independently controls virulence factor expression levels and export in *Yersinia* spp. *FEMS Microbiol Lett* **270**: 255–264.

Rott R, Zipor G, Portnoy V, Liveanu V & Schuster G (2003) RNA polyadenylation and degradation in cyanobacteria are similar to the chloroplast but different from *Escherichia coli*. *J Biol Chem* **278**: 15771–15777.

Rougemaille M, Villa T, Gudipati RK & Libri D (2008) mRNA journey to the cytoplasm: attire required. *Biol Cell* **100**: 327–342.

Sakai T, Nakamura N, Umitsuki G, Nagai K & Wachi M (2007) Increased production of pyruvic acid by *Escherichia coli* RNase G mutants in combination with *cra* mutations. *Appl Microbiol Biot* **76**: 183–192.

Sanson B & Uzan M (1995) Post-transcriptional controls in bacteriophage T4: roles of the sequence-specific endoribonuclease RegB. *FEMS Microbiol Rev* **17**: 141–150.

Santos JM, Drider D, Marujo PE, Lopez P & Arraiano CM (1997) Determinant role of *E. coli* RNase III in the decay of both specific and heterologous mRNAs. *FEMS Microbiol Lett* **157**: 31–38.

Schaeffer D, Tsanova B, Barbas A *et al.* (2009) The exosome contains domains with specific endoribonuclease, exoribonuclease and cytoplasmic mRNA decay activities. *Nat Struct Mol Biol* **16**: 56–62.

Schiffer S, Rosch S & Marchfelder A (2002) Assigning a function to a conserved group of proteins: the tRNA 3′-processing enzymes. *EMBO J* **21**: 2769–2777.

Schilling O, Ruggeberg S, Vogel A *et al.* (2004) Characterization of an *Escherichia coli elaC* deletion mutant. *Biochem Bioph Res Co* **320**: 1365–1373.

Seif E & Altman S (2008) RNase P cleaves the adenine riboswitch and stabilizes *pbuE* mRNA in *Bacillus subtilis*. *RNA* **14**: 1237–1243.

Sello JK & Buttner MJ (2008) The oligoribonuclease gene in *Streptomyces coelicolor* is not transcriptionally or translationally coupled to *adp*A, a key *bld*A target. *FEMS Microbiol Lett* **286**: 60–65.

Shahbabian K, Jamalli A, Zig L & Putzer H (2009) RNase Y, a novel endoribonuclease, initiates riboswitch turnover in *Bacillus subtilis*. *EMBO J* **28**: 3523–3533.

Shehi E, Serina S, Fumagalli G *et al.* (2001) The Sso7d DNA-binding protein from *Sulfolobus solfataricus* has ribonuclease activity. *FEBS Lett* **497**: 131–136.

Shi Z, Yang WZ, Lin-Chao S, Chak KF & Yuan HS (2008) Crystal structure of *Escherichia coli* PNPase: central channel residues are involved in processive RNA degradation. *RNA* **14**: 2361–2371.

Shibata HS, Minagawa A, Takaku H, Takagi M & Nashimoto M (2006) Unstructured RNA is a substrate for tRNase Z. *Biochemistry* **45**: 5486–5492.

Shiraishi H & Shimura Y (1986) Mutations affecting two distinct functions of the RNA component of RNase P. *EMBO J* **5**: 3673–3679.

Shyu AB, Wilkinson MF & van Hoof A (2008) Messenger RNA regulation: to translate or to degrade. *EMBO J* **27**: 471–481.

Sim SH, Yeom JH, Shin C *et al.* (2010) *Escherichia coli* ribonuclease III activity is downregulated by osmotic stress: consequences for the degradation of *bdm* mRNA in biofilm formation. *Mol Microbiol* **75**: 413–425.

Slomovic S, Laufer D, Geiger D & Schuster G (2005) Polyadenylation and degradation of human mitochondrial RNA: the prokaryotic past leaves its mark. *Mol Cell Biol* **25**: 6427–6435.

Slomovic S, Portnoy V, Yehudai-Resheff S, Bronshtein E & Schuster G (2008) Polynucleotide phosphorylase and the archaeal exosome as poly(A)-polymerases. *Biochim Biophys Acta* **1779**: 247–255.

Smith D, Burgin AB, Haas ES & Pace NR (1992) Influence of metal ions on the ribonuclease P reaction. Distinguishing substrate binding from catalysis. *J Biol Chem* **267**: 2429–2436.

Smith JK, Hsieh J & Fierke CA (2007) Importance of RNA–protein interactions in bacterial ribonuclease P structure and catalysis. *Biopolymers* **87**: 329–338.

Söderbom F & Wagner EG (1998) Degradation pathway of *Cop*A, the antisense RNA that controls replication of plasmid R1. *Microbiology* **144**: 1907–1917.

Söderbom F, Binnie U, Masters M & Wagner EG (1997) Regulation of plasmid R1 replication: PcnB and RNase E expedite the decay of the antisense RNA, *Cop*A. *Mol Microbiol* **26**: 493–504.

Sogin ML & Pace NR (1974) *In vitro* maturation of precursors of 5S ribosomal RNA from *Bacillus subtilis*. *Nature* **252**: 598–600.

Sogin ML, Pace B & Pace NR (1977) Partial purification and properties of a ribosomal RNA maturation endonuclease from *Bacillus subtilis*. *J Biol Chem* **252**: 1350–1357.

Sohlberg B, Huang J & Cohen SN (2003) The *Streptomyces coelicolor* polynucleotide phosphorylase homologue, and not the putative poly(A) polymerase, can polyadenylate RNA. *J Bacteriol* **185**: 7273–7278.

Song JJ, Smith SK, Hannon GJ & Joshua-Tor L (2004) Crystal structure of Argonaute and its implications for RISC slicer activity. *Science* **305**: 1434–1437.

Sousa S, Marchand I & Dreyfus M (2001) Autoregulation allows *Escherichia coli* RNase E to adjust continuously its synthesis to that of its substrates. *Mol Microbiol* **42**: 867–878.

Spickler C & Mackie GA (2000) Action of RNase II and polynucleotide phosphorylase against RNAs containing stem-loops of defined structure. *J Bacteriol* **182**: 2422–2427.

Stevens A & Niyogi SK (1967) Hydrolysis of oligoribonucleotides by an enzyme fraction from *Escherichia coli*. *Biochem Bioph Res Co* **29**: 550–555.

Stickney LM, Hankins JS, Miao X & Mackie GA (2005) Function of the conserved S1 and KH domains in polynucleotide phosphorylase. *J Bacteriol* **187**: 7214–7221.

Storz G, Opdyke JA & Zhang A (2004) Controlling mRNA stability and translation with small, noncoding RNAs. *Curr Opin Microbiol* **7**: 140–144.

Stougaard P, Molin S & Nordström K (1981) RNAs involved in copy-number control and incompatibility of plasmid R1. *P Natl Acad Sci USA* **78**: 6008–6012.

Subbarayan PR & Deutscher MP (2001) *Escherichia coli* RNase M is a multiply altered form of RNase I. *RNA* **7**: 1702–1707.

Sulewski M, Marchese-Ragona SP, Johnson KA & Benkovic SJ (1989) Mechanism of polynucleotide phosphorylase. *Biochemistry* **28**: 5855–5864.

Symmons MF, Jones GH & Luisi BF (2000) A duplicated fold is the structural basis for polynucleotide phosphorylase catalytic activity, processivity, and regulation. *Structure* **8**: 1215–1226.

Szczesny RJ, Borowski LS, Brzezniak LK, Dmochowska A, Gewartowski K, Bartnik E & Stepien PP (2010) Human mitochondrial RNA turnover caught in flagranti: involvement of hSuv3p helicase in RNA surveillance. *Nucleic Acids Res* **38**: 279–298.

Tadokoro T & Kanaya S (2009) Ribonuclease H: molecular diversities, substrate binding domains, and catalytic mechanism of the prokaryotic enzymes. *FEBS J* **276**: 1482–1493.

Taghbalout A & Rothfield L (2007) RNaseE and the other constituents of the RNA degradosome are components of the bacterial cytoskeleton. *P Natl Acad Sci USA* **104**: 1667–1672.

Taghbalout A & Rothfield L (2008) RNaseE and RNA helicase B play central roles in the cytoskeletal organization of the RNA degradosome. *J Biol Chem* **283**: 13850–13855.

Takaku H & Nashimoto M (2008) *Escherichia coli* tRNase Z can shut down growth probably by removing amino acids from aminoacyl-tRNAs. *Genes Cells* **13**: 1087–1097.

Takechi S, Yasueda H & Itoh T (1994) Control of ColE2 plasmid replication: regulation of Rep expression by a plasmid-coded antisense RNA. *Mol Gen Genet* **244**: 49–56.

Taraseviciene L, Miczak A & Apirion D (1991) The gene specifying RNase E (*rne*) and a gene affecting mRNA stability (*ams*) are the same gene. *Mol Microbiol* **5**: 851–855.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F & Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**: 4876–4882.

Tobe T, Sasakawa C, Okada N, Honma Y & Yoshikawa M (1992) *vacB*, a novel chromosomal gene required for expression of virulence genes on the large plasmid of *Shigella flexneri*. *J Bacteriol* **174**: 6359–6367.

Tock MR, Walsh AP, Carroll G & McDowall KJ (2000) The CafA protein required for the 5′-maturation of 16 S rRNA is a 5′-end-dependent ribonuclease that has context-dependent broad sequence specificity. *J Biol Chem* **275**: 8726–8732.

Tomecki R, Dmochowska A, Gewartowski K, Dziembowski A & Stepien PP (2004) Identification of a novel human nuclear-encoded mitochondrial poly(A) polymerase. *Nucleic Acids Res* **32**: 6001–6014.

Turnbough CL Jr, Kerr KH, Funderburg WR, Donahue JP & Powell FE (1987) Nucleotide sequence and characterization of the *pyr*F operon of *Escherichia coli* K12. *J Biol Chem* **262**: 10239–10245.

Udekwu KI, Darfeuille F, Vögel J, Reimegard J, Holmqvist E & Wagner EG (2005) Hfq-dependent regulation of OmpA synthesis is mediated by an antisense RNA. *Gene Dev* **19**: 2355–2366.

Umitsuki G, Wachi M, Takada A, Hikichi T & Nagai K (2001) Involvement of RNase G in *in vivo* mRNA metabolism in *Escherichia coli*. *Genes Cells* **6**: 403–410.

Uzan M (2001) Bacteriophage T4 RegB endoribonuclease. *Method Enzymol* **342**: 467–480.

Uzan M (2009) RNA processing and decay in bacteriophage T4. *Prog Mol Biol Transl Sci* **85**: 43–89.

van Hoof A & Parker R (1999) The exosome: a proteasome for RNA? *Cell* **99**: 347–350.

Vanzo NF, Li YS, Py B *et al.* (1998) Ribonuclease E organizes the protein interactions in the *Escherichia coli* RNA degradosome. *Gene Dev* **12**: 2770–2781.

Viegas SC & Arraiano CM (2008) Regulating the regulators: How ribonucleases dictate the rules in the control of small non-coding RNAs. *RNA Biol* **5**: 230–243.

Viegas SC, Schmidt D, Kasche V, Arraiano CM & Ignatova Z (2005) Effect of the increased stability of the penicillin amidase mRNA on the protein expression levels. *FEBS Lett* **579**: 5069–5073.

Viegas SC, Pfeiffer V, Sittka A, Silva IJ, Vogel J & Arraiano CM (2007) Characterization of the role of ribonucleases in *Salmonella* small RNA decay. *Nucleic Acids Res* **35**: 7651–7664.

Vincent HA & Deutscher MP (2006) Substrate recognition and catalysis by the exoribonuclease RNase R. *J Biol Chem* **281**: 29769–29775.

Vincent HA & Deutscher MP (2009) The roles of individual domains of RNase R in substrate binding and exoribonuclease activity. The nuclease domain is sufficient for digestion of structured RNA. *J Biol Chem* **284**: 486–494.

Viswanathan M, Dower KW & Lovett ST (1998) Identification of a potent DNase activity associated with RNase T of *Escherichia coli*. *J Biol Chem* **273**: 35126–35131.

Vogel A, Schilling O, Niecke M, Bettmer J & Meyer-Klaucke W (2002) ElaC encodes a novel binuclear zinc phosphodiesterase. *J Biol Chem* **277**: 29078–29085.

Vogel J & Hess WR (2001) Complete 5′ and 3′ end maturation of group II intron-containing tRNA precursors. *RNA* **7**: 285–292.

Vogel J, Argaman L, Wagner EG & Altuvia S (2004) The small RNA IstR inhibits synthesis of an SOS-induced toxic peptide. *Curr Biol* **14**: 2271–2276.

von Gabain A, Belasco JG, Schottel JL, Chang AC & Cohen SN (1983) Decay of mRNA in *Escherichia coli*: investigation of the fate of specific segments of transcripts. *P Natl Acad Sci USA* **80**: 653–657.

Wachi M, Umitsuki G, Shimizu M, Takada A & Nagai K (1999) *Escherichia coli cafA* gene encodes a novel RNase, designated as RNase G, involved in processing of the 5′ end of 16S rRNA. *Biochem Bioph Res Co* **259**: 483–488.

Wagner EG, Altuvia S & Romby P (2002) Antisense RNAs in bacteria and their genetic elements. *Adv Genet* **46**: 361–398.

Wagner S & Klug G (2007) An archaeal protein with homology to the eukaryotic translation initiation factor 5A shows ribonucleolytic activity. *J Biol Chem* **282**: 13966–13976.

Wang W & Bechhofer DH (1996) Properties of a *Bacillus subtilis* polynucleotide phosphorylase deletion strain. *J Bacteriol* **178**: 2375–2382.

Wang W & Bechhofer DH (1997) *Bacillus subtilis* RNase III gene: cloning, function of the gene in *Escherichia coli*, and construction of *Bacillus subtilis* strains with altered *rnc* loci. *J Bacteriol* **179**: 7379–7385.

Wen T, Oussenko IA, Pellegrini O, Bechhofer DH & Condon C (2005) Ribonuclease PH plays a major role in the exonucleolytic maturation of CCA-containing tRNA precursors in *Bacillus subtilis*. *Nucleic Acids Res* **33**: 3636–3643.

Willkomm DK, Feltens R & Hartmann RK (2002) tRNA maturation in *Aquifex aeolicus*. *Biochimie* **84**: 713–722.

Winkler WC & Breaker RR (2005) Regulation of bacterial gene expression by riboswitches. *Annu Rev Microbiol* **59**: 487–517.

Worrall JA & Luisi BF (2007) Information available at cut rates: structure and mechanism of ribonucleases. *Curr Opin Struc Biol* **17**: 128–137.

Worrall JA, Gorna M, Crump NT *et al.* (2008a) Reconstitution and analysis of the multienzyme *Escherichia coli* RNA degradosome. *J Mol Biol* **382**: 870–883.

Worrall JA, Howe FS, McKay AR, Robinson CV & Luisi BF (2008b) Allosteric activation of the ATPase activity of the *Escherichia coli* RhlB RNA helicase. *J Biol Chem* **283**: 5567–5576.

Wu J, Jiang Z, Liu M, Gong X, Wu S, Burns CM & Li Z (2009) Polynucleotide phosphorylase protects *Escherichia coli* against oxidative stress. *Biochemistry* **48**: 2012–2020.

Xu A, Jao DL & Chen KY (2004) Identification of mRNA that binds to eukaryotic initiation factor 5A by affinity co-purification and differential display. *Biochem J* **384**: 585–590.

Xu F & Cohen SN (1995) RNA degradation in *Escherichia coli* regulated by 3′ adenylation and 5′ phosphorylation. *Nature* **374**: 180–183.

Xu F, Lin-Chao S & Cohen SN (1993) The *Escherichia coli pcn*B gene promotes adenylylation of antisense RNAI of ColE1-type plasmids *in vivo* and degradation of RNAI decay intermediates. *P Natl Acad Sci USA* **90**: 6756–6760.

Yamaguchi Y & Inouye M (2009) mRNA interferases, sequence-specific endoribonucleases from the toxin-antitoxin systems. *Prog Mol Biol Transl Sci* **85**: 467–500.

Yang L & Altman S (2007) A noncoding RNA in *Saccharomyces cerevisiae* is an RNase P substrate. *RNA* **13**: 682–690.

Yang W, Hendrickson WA, Crouch RJ & Satow Y (1990) Structure of ribonuclease H phased at 2 Å resolution by MAD analysis of the selenomethionyl protein. *Science* **249**: 1398–1405.

Yao S, Blaustein JB & Bechhofer DH (2007) Processing of *Bacillus subtilis* small cytoplasmic RNA: evidence for an additional endonuclease cleavage site. *Nucleic Acids Res* **35**: 4464–4473.

Yehudai-Resheff S, Hirsh M & Schuster G (2001) Polynucleotide phosphorylase functions as both an exonuclease and a poly(A) polymerase in spinach chloroplasts. *Mol Cell Biol* **21**: 5408–5416.

Zangrossi S, Briani F, Ghisotti D, Regonesi ME, Tortora P & Dehò G (2000) Transcriptional and post-transcriptional control of polynucleotide phosphorylase during cold acclimation in *Escherichia coli*. *Mol Microbiol* **36**: 1470–1480.

Zhang JR & Deutscher MP (1988a) Cloning, characterization, and effects of overexpression of the *Escherichia coli rnd* gene encoding RNase D. *J Bacteriol* **170**: 522–527.

Zhang JR & Deutscher MP (1988b) Transfer RNA is a substrate for RNase D *in vivo*. *J Biol Chem* **263**: 17909–17912.

Zhang JR & Deutscher MP (1989) Analysis of the upstream region of the *Escherichia coli rnd* gene encoding RNase D. Evidence for translational regulation of a putative tRNA processing enzyme. *J Biol Chem* **264**: 18228–18233.

Zhang K & Nicholson AW (1997) Regulation of ribonuclease III processing by double-helical sequence antideterminants. *P Natl Acad Sci USA* **94**: 13437–13441.

Zhang X, Zhu L & Deutscher MP (1998) Oligoribonuclease is encoded by a highly conserved gene in the 3′-5′ exonuclease superfamily. *J Bacteriol* **180**: 2779–2781.

Zhu LQ, Gangopadhyay T, Padmanabha KP & Deutscher MP (1990) *Escherichia coli rna* gene encoding RNase I: cloning, overexpression, subcellular distribution of the enzyme, and use of an *rna* deletion to identify additional RNases. *J Bacteriol* **172**: 3146–3151.

Zilhão R, Camelo L & Arraiano CM (1993) DNA sequencing and expression of the gene *rnb* encoding *Escherichia coli* ribonuclease II. *Mol Microbiol* **8**: 43–51.

Zilhão R, Caillet J, Regnier P & Arraiano CM (1995a) Precise physical mapping of the *Escherichia coli rnb* gene, encoding ribonuclease II. *Mol Gen Genet* **248**: 242–246.

Zilhão R, Régnier P & Arraiano CM (1995b) The role of endonucleases in the expression of ribonuclease II in *Escherichia coli*. *FEMS Microbiol Lett* **130**: 237–244.

Zilhão R, Cairrão F, Régnier P & Arraiano CM (1996a) PNPase modulates RNase II expression in *Escherichia coli*: implications for mRNA decay and cell metabolism. *Mol Microbiol* **20**: 1033–1042.

Zilhão R, Plumbridge J, Hajnsdorf E, Regnier P & Arraiano CM (1996b) *Escherichia coli* RNase II: characterization of the promoters involved in the transcription of *rnb*. *Microbiology* **142**: 367–375.

Zuo Y & Deutscher MP (2001) Exoribonuclease superfamilies: structural analysis and phylogenetic distribution. *Nucleic Acids Res* **29**: 1017–1026.

Zuo Y & Deutscher MP (2002) The physiological role of RNase T can be explained by its unusual substrate specificity. *J Biol Chem* **277**: 29654–29661.

Zuo Y, Wang Y & Malhotra A (2005) Crystal structure of *Escherichia coli* RNase D, an exoribonuclease involved in structured RNA processing. *Structure* **13**: 973–984.

Zuo Y, Vincent HA, Zhang J, Wang Y, Deutscher MP & Malhotra A (2006) Structural basis for processivity and single-strand specificity of RNase II. *Mol Cell* **24**: 149–156.

Zuo Y, Zheng H, Wang Y *et al*. (2007) Crystal structure of RNase T, an exoribonuclease involved in tRNA maturation and end turnover. *Structure* **15**: 417–428.

Biochemical and Biophysical Research Communications 411 (2011) 50–55

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A new target for an old regulator: H-NS represses transcription of *bolA* morphogene by direct binding to both promoters

Ricardo N. Moreira, Clémentine Dressaire, Susana Domingues, Cecília M. Arraiano *

Instituto de Tecnologia Química e Biológica/Universidade Nova de Lisboa, Apartado 127, 2781-901 Oeiras, Portugal

ARTICLE INFO

Article history:
Received 24 May 2011
Available online 17 June 2011

Keywords:
Transcriptional regulator
Stress conditions
DNA binding
σ^s-Regulated gene
H-NS
bolA

ABSTRACT

The *Escherichia coli bolA* morphogene is very important in adaptation to stationary phase and stress response mechanisms. Genes of this family are widespread in gram negative bacteria and in eukaryotes. The expression of this gene is tightly regulated at transcriptional and post-transcriptional levels and its overexpression is known to induce round cellular morphology. The results presented in this report demonstrate that the H-NS protein, a pleiotropic regulator of gene expression, is a new transcriptional modulator of the *bolA* gene. In this work we show that *in vivo* the levels of *bolA* are down-regulated by H-NS and *in vitro* this global regulator interacts directly with the *bolA* promoter region. Moreover, DNaseI footprinting experiments mapped the interaction regions of H-NS and *bolA* and revealed that this global regulator binds not only one but both *bolA* promoters. We provide a new insight into the *bolA* regulation network demonstrating that H-NS represses the transcription of this important gene.

1. Introduction

The *Escherichia coli bolA* gene is induced at the onset of stationary phase and in response to many forms of stress [1]. The overexpression of *bolA* leads to substantial changes in the cell and the bacterial bacilli transform into spheres [1–3]. The fact that BolA affects the expression of numerous genes highlights its importance, and previous reports show that *bolA* can act as a transcription factor. For instance, BolA has been demonstrated to specifically interact with the *mreB* promoter, repressing its transcription [4]. This leads to a reduction in MreB protein levels and consequently to an abnormal MreB polymerization. BolA was also shown to directly regulate the transcript levels of the important D,D-carboxypeptidases PBP5 and PBP6, and to modulate the expression levels of the β-lactamase *ampC* [2,5]. Furthermore, *bolA* is involved in biofilm development and promotes changes in the outer membrane that affect permeability and resistance to antibiotics such as vancomycin [6,7]. It is not surprising that the expression of a gene involved in the control of several cellular processes is tightly regulated at transcriptional and post-transcriptional levels. In optimal growth conditions, *bolA* is under the control of a weak σ^{70}-dependent constitutive promoter, *bolAp2*. During stress and stationary phase it is mostly transcribed from the strong gearbox promoter, *bolAp1*, induced by the sigma factor σ^s [3,8]. As a σ^s-regulated gene, *bolA* expression is sensitive to ppGpp [9] and cAMP [10] intracellular levels. *bolA* was also shown to be repressed by the direct binding of OmpR in its phosphorylated form [11]. Ribonuclease III (RNase III) and polyA polymerase (PAPI) are involved in post-transcriptional control of *bolA* expression [12–14].

The histone-like (or heat-stable) protein H-NS was shown to affect some σ^s-dependent genes [15]. This 15 kDa nucleoid-associated protein is abundant in bacterial cells and is often compared to eukaryotic histones because of its high affinity for DNA. It binds preferentially to curved AT-rich regions that are found in certain promoter regions [16].

In this work we show that H-NS down-regulates *bolA* levels. We demonstrate that this regulation is mediated by a specific binding of H-NS to the *bolA* promoter region, involving both promoters. The interaction region of H-NS with *bolA* promoters was mapped and the implications of *bolA* regulation by H-NS are discussed.

2. Materials and methods

Restriction enzymes, T4 DNA ligase, Pfu DNA polymerase and T4 polynucleotide kinase were purchased from Fermentas. DNaseI was purchased from Sigma. All the enzymes were used according to the supplier's instructions. Oligonucleotide primers used in this work are listed in Table 1 and were synthesized by STAB Vida, Portugal.

2.1. Bacterial strains and plasmids

The *E. coli* strains used were: DH5α (F′ *fhuA2* Δ(*argF-lacZ*)*U169* phoA glnV44 Φ80 Δ(*lacZ*)*M15* gyrA96 recA1 relA1 endA1 thi-1 hsdR17a) for cloning experiments; BL21(DE3) ($F^- r_B^- m_B^-$ *gal ompT* (*int::*P_{lacUV5} T7 gen1 imm21 nin5) for overexpression and

* Corresponding author. Fax: +351 214469549.
E-mail address: cecilia@itqb.unl.pt (C.M. Arraiano).

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.06.084

Table 1
Oligonucleotides used in this work.

Oligonucleotides	Sequence
hnsNdeI	5′-GGAATTCCATATGAGCGAAGCACTTAAAATTCTG-3′
hnsBamHI	5′-CGGGATCCCGTTATTGCTTGATCAGGAAATCGTCGAGGG-3′
X2	5′-GTCACAATGTCCCAGCCG-3′
X7	5′-CGATGCTTCCTGCTCCAC-3′
16sF	5′-AGAGTTTGATCCTGGCTCAG-3′
16sR	5′-ACGGCTACCTTGTTACGACTT-3′
bolAFw	5′-GGGGTACCTGTTTGGTAAAAATTCCCG-3′
RNM012	5′-TCTATCCGCTCACGTATCAT-3′
RblrealT	5′-AGTTCCTCCGCTAAAGTACTG-3′
P2	5′-CTTGACGGAAAAACCAGGACG-3′
FblrealT	5′-AACCCGTATTCCTCGAAGTAG-3′

purification of the H-NS protein; JW1225 (Δ*hns::kan*^{r}) [17]; MG1655; MG1693 (a spontaneous Thy$^-$ derivative of strain MG1655); and CMA92 (MG1655 Δ*hns::kan*^{r}), this work. These strains were grown in Luria Broth medium (LB) at 37 °C, supplemented with 100 μg/ml ampicillin or 50 μg/ml kanamycin, when required.

The *hns* coding sequence was amplified by PCR using *E. coli* MG1693 chromosomal DNA and the primers hnsNdeI and hnsBamHI. The amplified fragment was cut with NdeI and BamHI restriction enzymes and cloned into the pET-15b vector (Novagen) previously cleaved with the same enzymes. The resulting plasmid (pCDA1) encoding H-NS fused to an N-terminal His6-tag was used to transform *E. coli* BL21(DE3) resulting in CMA93 strain (BL21(DE3) + pCDA1).

The *hns* deletion mutant was obtained from the Keio collection [17]. P1-mediated transduction to transfer the mutation to the MG1655 background (CMA92) was performed as previously described [18]. All constructions were confirmed by DNA sequencing at STAB Vida, Portugal.

2.2. Expression and purification of H-NS

BL21(DE3) containing pCDA1 was grown overnight at 37 °C, 120 rpm in LB media supplemented with 100 $\mu g \cdot ml^{-1}$ ampicillin. Fresh 250 ml of LB was inoculated with the overnight culture to a final OD_{600} of 0.1 and the culture was incubated at 37 °C, 180 rpm. At OD_{600} – 0.5, the expression of *hns* was induced with 1 mM IPTG for 2 h in the same growing conditions. Cells were harvested by centrifugation and the pellets stored at −80 °C. The cellular pellets were resuspended in 6 ml of buffer A (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4) supplemented with 0.1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were then disrupted using a French press at 9000 psi and the crude extracted was treated with Benzonase (Sigma) to degrade the nucleic acids. After 30 min incubation on ice, the suspension was centrifuged for 30 min, at 48,000*g*, 4 °C. The supernatant was collected and loaded into a HisTrap Chelating Sepharose 1 ml column (GE Healthcare) equilibrated in buffer A using an AKTA HLPC system (GE Healthcare). Elution was performed using a gradient of buffer B (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4) from 0% to 100% in 20 min. Collected fractions containing the pure protein were pooled together and buffer exchanged to buffer C (20 mM sodium phosphate, 0.5 M NaCl, pH 7.4) using a desalting 5 ml column (GE Healthcare). Eluted proteins were then concentrated by centrifugation at 4 °C with an Amicon Ultra Centrifugal Filters Devices (Millipore) with a mass cutoff of 10 kDa. Protein concentration was determined by the Bradford quantification method and 50% (v/v) glycerol was added to the final fractions prior to storage at −20 °C. More than 90% homogeneity was revealed by analyzing the purified protein in a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) stained with Coomassie blue.

2.3. RNA extraction and RT-PCR

Total RNA was extracted by the Trizol (Ambion) according to the supplier instructions with some modifications. Briefly, an overnight CMA92 culture was diluted to a final OD_{600} of 0.1 and incubated at 37 °C, 180 rpm. Samples were collected at different points corresponding to the different phases of the bacterial growth curve (exponential – 0.4; late exponential – 1.2; and stationary phase – 2.5). Each aliquot containing 20 ml of bacterial cell culture was mixed with an equal volume of TM buffer (10 mM Tris, 25 mM NaN_3, 500 μg/ml chloramphenicol, 5 mM $MgCl_2$, pH 7.2) and harvested by centrifugation. The cell pellet was resuspended in 600 μl of lysis buffer (10 mM Tris, 5 mM $MgCl_2$, 300 μg/ml lysozyme, pH 7.2) followed by five cycles of freeze and thaw. The suspension was supplemented with 1% SDS and 0.33 mM AcOH. One milliliter of Trizol reagent (Ambion) was added and the suspension was vortexed 5 min at room temperature, followed by a 10 min centrifugation at 16000*g*, 4 °C. The aqueous phase was collected and mixed with 200 μl of chloroform. The mixture was vortexed again for 15 min at room temperature and centrifuged for 15 min at 4 °C. The aqueous phase was collected and total RNA was precipitated with isopropanol. After drying, the pellet was resuspended in H_2O and the RNA concentration was measured with a spectrophotometer (NanoDrop 1000).

Reverse transcription-PCR (RT-PCR) was carried out with 50 ng of total RNA, with the OneStep RT-PCR kit (Qiagen), according to the supplier's instructions, using oligonucleotides X2 and X7. As an independent control, the 16S rRNA-specific primers 16sF and 16sR were used. Prior to RT-PCR, all RNA samples were treated with Turbo DNA free Kit (Ambion). Control experiments, run in the absence of reverse transcriptase, yielded no product.

2.4. Electrophoretic mobility shift assays

All the fragments used in the electrophoretic mobility shift assays (EMSA) experiments were generated by PCR and were radioactively labeled at their 5′-end. For this purpose the reverse primer in each PCR reaction was previously end-labeled with [^{32}P]-γ-ATP using T4 polynucleotide kinase. PCR reactions were carried out using genomic DNA from *E. coli* MG1693 as template. Four different substrates were obtained with different primer pairs: bolAFw and RNM012; bolAFw and RblrealT; P2 and RblrealT; FblrealT and RbrealT. The resulting PCR fragments were run in a 5% non-denaturing polyacrylamide (PAA) gel and purified by the crush and soak method previously described [18]. The concentration of the purified fragments was measured in a Biophotometer Plus (Eppendorf).

Binding reactions were performed in a total volume of 10 μl containing EMSA buffer (10 mM Tris–HCl pH 8, 10 mM $MgCl_2$, 100 mM NaCl, 10 mM KCl, 0.5 mM DTT, 5% glycerol), 1 nM of labeled substrate and increasing concentrations of purified H-NS. H-NS was diluted to the desired concentrations prior to the assay in 2 mM Tris–HCl pH 8, 0.2 mM DTT, 10 mM KCl and 10 mM NaCl. In all the assays a control reaction without protein was performed. The binding reactions were incubated at room temperature for 20 min and the samples were then analyzed in a 5% non-denaturing PAA gel. DNA–protein complexes were detected using the PhosphorImager system from Molecular Dynamics.

2.5. DNaseI footprinting

DNaseI footprinting assays were performed as described by Leblanc and Moss [19] with some modifications. Briefly, the DNA–protein complexes obtained as described above (but in a total volume of 50 μl), were supplemented with a cofactor solution (5 mM $CaCl_2$, 10 mM $MgCl_2$) and 5×10^{-3} Kunitz units/μL of DNaseI, and incubated 2 min at room temperature. The digestion reaction

was stopped with addition of stop buffer (1% SDS, 200 mM NaCl, 20 mM EDTA, pH 8.0) followed by phenol-chloroform-isoamylalcohol (Sigma) extraction of the digested DNA. The extracted DNA was resuspended in formamide dye mix [95% deionized formamide, 0.025% (w/v) bromophenol blue, 0.025% (w/v) xylene cyanol FF, 5 mM EDTA pH 8.0, 0.025% (w/v) SDS], resolved in a 8% denaturing 8.3 M urea PAA sequencing gel at 1500 V in 1X TBE. Digested fragments were detected using a PhosphorImager system from Molecular Dynamics. M13 sequencing reaction was performed with Sequenase Version 2.0 sequencing kit according to the instructions manual and resolved in the same gel.

2.6. DNA curvature analysis

The online available DNA curvature analysis software (<http://www.lfd.uci.edu/~gohlke/dnacurve/>) was used with AA Wedge algorithm. This bioinformatics tool enables the compilation of the curvature values and the calculation of the global 3D structure of a DNA molecule from its nucleotide sequence. This program was used to obtain the 3D model of the *bolA* promoter region.

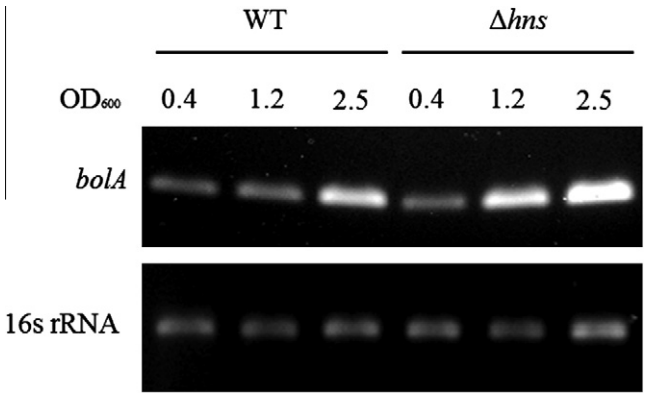

Fig. 1. Down-regulation of the *bolA* transcript level by H-NS. RT-PCR amplification of *bolA* transcript from total RNA extracted in different growth phases: *E. coli* exponential (OD_{600} 0.4), late exponential (OD_{600} 1.2) and stationary phase (OD_{600} 2.5) (upper image). Control experiments performed with 16s rRNA specific primers (image on the bottom) indicated that there were no significant differences in the amounts of RNA in each sample.

3. Results and discussion

3.1. Effect of H-NS in bolA expression

In optimal growth conditions during exponential phase *bolA* is regulated by σ^{70} and only relatively low mRNA levels are detected in the cell. However in stationary phase, *bolA* expression is under the control of σ^{s} and a 5-fold increase of the transcript level is observed [1]. Under stress conditions the *bolA* levels can increase further [1]. Since H-NS is a global regulator shown to affect the expression of several genes that respond to stress and are regulated by σ^{s}, we wanted to test whether H-NS could also be involved in the control of *bolA* expression. Taking into account that *bolA* is growth-phase regulated, the influence of H-NS on its expression was assayed during different phases of bacterial growth. Three points were analyzed (OD_{600} of 0.4, 1.2 and 2.5) corresponding to exponential, late exponential and stationary phase. For each optical density, samples were taken and total RNA was extracted from a WT cell culture and the isogenic Δ*hns* strain. *bolA* mRNA levels were then estimated by RT-PCR using a pair of specific primers (Fig. 1). In agreement with the previous results for the wild type strain, *bolA* levels are low during exponential phase and reach a maximum during stationary phase. In the absence of H-NS, the *bolA* levels in late exponential phase are significantly higher than in the wt and increase ~2.4-fold. In the *hns* strain there is also an increase of *bolA* in stationary

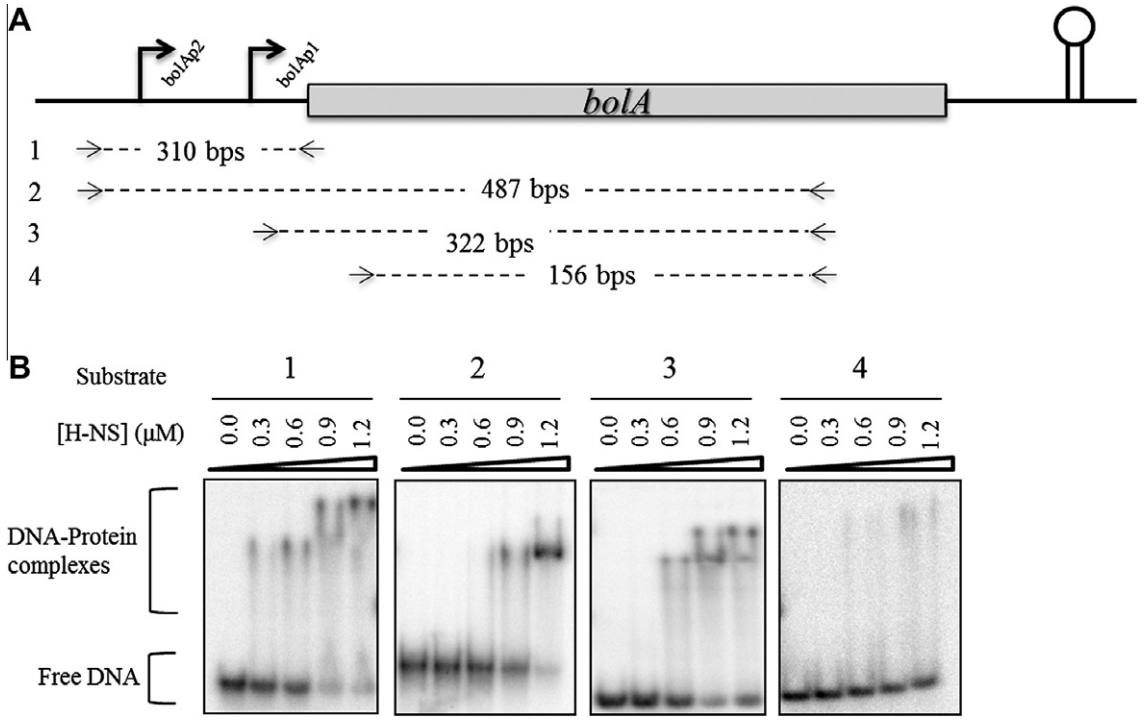

Fig. 2. DNA–protein interactions of *bolA* promoters and H-NS. (A) Schematic representation of *bolA* genomic region. The different substrates used in the electro-mobility shift assays (EMSA) are represented. (B) Representative EMSA of H-NS with 1 nM of the indicated substrates above the respective image. A control reaction without protein ([H-NS] = 0.0 μM) was performed in all experiments. Binding reactions using an increasing concentration of H-NS (indicated at the top of each lane) were resolved in a 5% non-denaturing polyacrylamide gel. Free DNA and DNA–protein complexes are indicated.

phase but the difference is quite lower. H-NS is probably repressing the expression of *bolA* during late exponential growth similarly to what happens when it regulates the *hchA* gene [20]. On the other hand, in stationary phase competes with the higher σ^s levels in the cell, and that is probably why it can not exert the same level of repression.

Taken together, these results indicate that H-NS downregulates *bolA* expression, supporting our hypothesis that H-NS could be a transcriptional repressor of *bolA* expression. Moreover, these results provide another evidence for the key function of H-NS as a selective silencer of genes that rapidly respond to environmental changes [15,21,22].

3.2. H-NS binds specifically to bolA promoter(s)

The RT-PCR results indicate that H-NS is involved in the modulation of *bolA* mRNA levels. However the nature of this regulation remains unknown. H-NS is known to be able to modulate gene expression in at least two different ways: by directly binding to specific targets or indirectly through the down-regulation of the σ^s transcript [15]. Thus we tested if H-NS could be acting directly over *bolA* as a transcriptional regulator. For this purpose, the *E. coli* H-NS protein was purified near homogeneity (Figure S1) and the pure protein was used in gel mobility shift assays with the *bolA* genomic region. Four different substrates were used in order to discriminate the ability of H-NS to bind the *bolA* upstream region (Fig. 2A). As a protein that binds DNA with high affinity, H-NS was able to retard the mobility of all the DNA fragments tested, generating retardation bands that correspond to DNA–protein complexes (Fig. 2B). However, some relevant differences were observed among the substrates tested. The substrate 1, comprising only *bolAp2* and *bolAp1* is clearly the preferred H-NS substrate. For this substrate DNA–protein complexes could be observed with only 0.3 μM of H-NS, while at least a 2-fold excess was needed for the formation of DNA–protein complexes with any of the other substrates. In addition, when using substrate 1 almost all DNA was bound with only 0.9 μM of H-NS, whereas the amount of protein needed to completely bind the substrate 2 (*bolAp2* + *bolAp1* + ORF) raised to 1.2 μM. This amount of H-NS was not even sufficient to completely bind substrate 3 (missing *bolAp2*), and at this protein concentration free DNA was still detected. At higher H-NS concentrations, a retarded band of higher molecular mass could be detected. The appearance of this band was concomitant with the disappearance of the complex of lower mass. The higher band probably corresponds to the binding of more than one molecule per substrate. H-NS is indeed known to form higher order structure complexes with its targets [23]. With substrate 1, at 1.2 μM almost all DNA molecules seem to be bound by more than one protein molecule. When using substrate 2 with the same H-NS concentration, this higher order complex is almost absent, indicating that the majority of DNA is still bound by only one H-NS molecule. The substrate missing *bolAp2* presents an intermediate situation since both protein complexes are equally detected. These experiments show that *in vitro* the presence of the whole *bolA* coding region (substrate 2) or the deletion of *bolAp2* (substrate 3) seems to affect the efficiency of the H-NS binding to *bolA*. Finally, H-NS was also able to bind to the substrate which comprises only the *bolA* coding region (substrate 4), although with a significantly lower affinity. Together, these results suggest that H-NS binds preferentially the *bolA* promoter region (with both promoters). H-NS is not only sequence but also structure sensitive [21]. Despite *bolAp1* and *bolAp2* being present in substrate 2, the additional presence of the *bolA* ORF may change the conformation of the promoter region (see below), thus affecting H-NS binding. This likely explains the partial loss of H-NS binding affinity for the longer substrate.

These experiments clearly show that the efficiency of H-NS binding is affected by both *bolA* promoters. Together with the *in vivo* data these results provide strong evidence that H-NS represses *bolA* transcription through a direct interaction with the entire *bolA* promoter region. Our results indicate that the reported co-immunoprecipitation of *bolA* with H-NS [16] was probably due to a direct interaction with this *bolA* region.

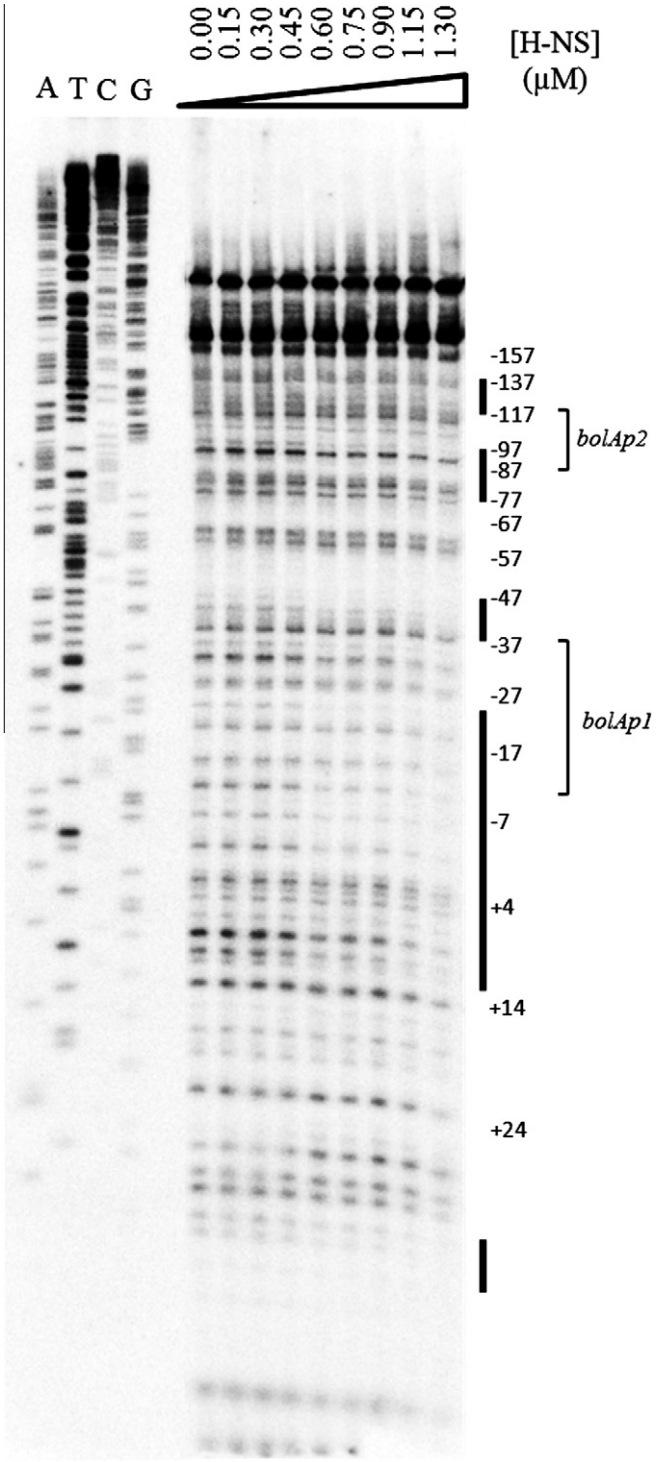

Fig. 3. Mapping of H-NS binding sites on *bolA* promoters by DNaseI foot-printing. Electrophoretic separation of a fragment with *bolAp2* and *bolAp1* after H-NS binding followed by DNaseI digestion. The different lanes correspond to different H-NS concentrations, as indicated on top. The lanes labeled with A, T, C and G represent the M13 sequencing reaction. The sites that are protected by H-NS are indicated with black lines. The numbers indicate nucleotide positions with respect to the *bolAp1* transcription start site.

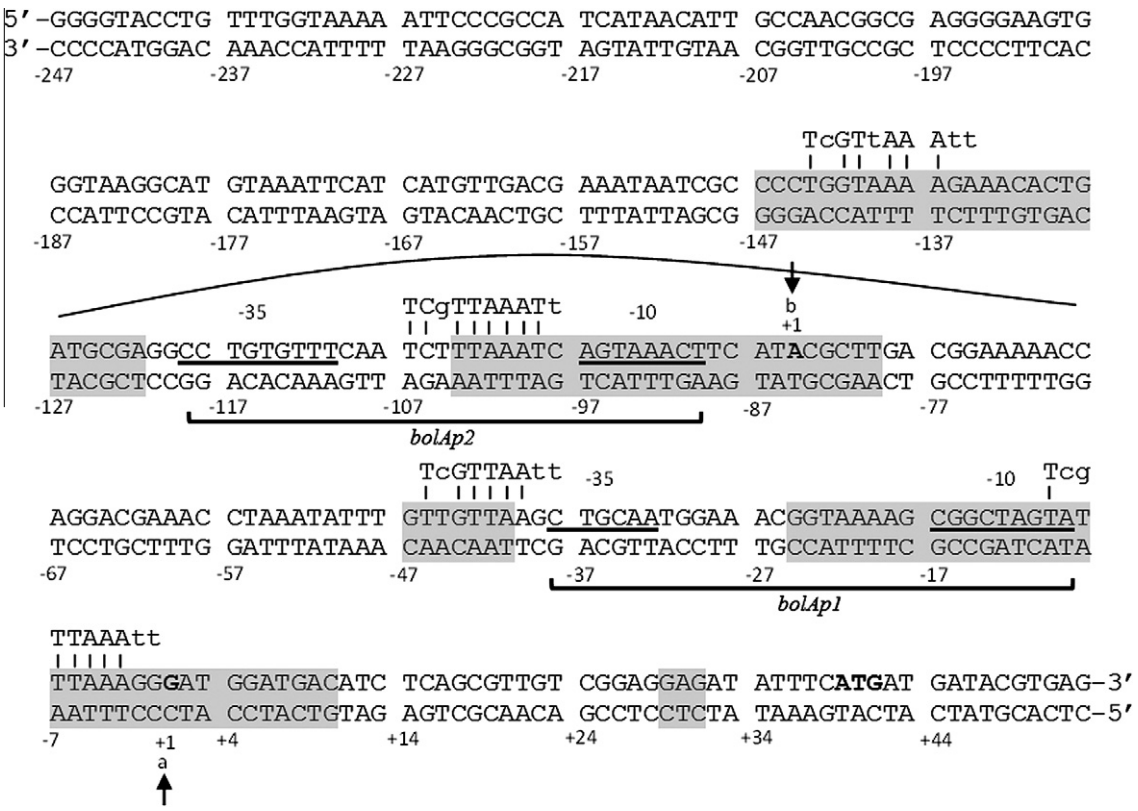

Fig. 4. Schematic representation of *bolA* promoter region. The numbers indicate nucleotide positions with respect to the *bolAp1* transcription start site. The sites revealed by H-NS-mediated DNaseI protection are highlighted in gray and the -35 and -10 regions of the promoters are underlined. The transcription start sites (a and b), and the initiation codon are in bold. The H-NS-binding consensus sequence is indicated above the DNA duplex, and the vertical bars indicate the base match between consensus and promoter sequence. The region of DNA predicted to have significant curvature is indicated by a curved line immediately above the sequence.

3.3. DNA curvature of bolA promoter region

DNA curvature analysis bioinformatics tool was used to calculate the bending region(s) to evaluate if 3D structure of the *bolA* promoters could influence H-NS binding. The double helix of a DNA fragment containing both *bolA* promoters displayed an accentuated curvature, possibly favoring the interaction with H-NS (Figure S2). However, when a DNA fragment lacking *bolAp2* was analyzed (such as substrate 3), the curvature is close to null. Hence, the DNA curvature seems to be directly dependent on the *bolAp2* region. Since the DNA structure is an important factor for the proper H-NS binding specificity, this could be one reason for the variations observed above (in the gel retardation assays).

3.4. Mapping of H-NS interaction sites

To pursue our studies and clearly identify the region(s) of interaction between H-NS and the *bolA* promoters we have performed DNaseI foot-printing assays to map the H-NS binding sites to the *bolA* promoters. We used a DNA fragment containing both *bolAp2* and *bolAp1* (substrate 1 – Fig. 2) and protection zones were identified (Fig. 3). The interaction regions were evenly distributed through the entire region analyzed, which demonstrates that H-NS can bind to several sites covering both *bolA* promoters. Sequence analysis demonstrated that the largest protection site was detected in the gearbox promoter *bolAp1* (Fig. 4). A narrower protection zone was found upstream of the -35 box of this promoter. Two other main interaction regions were mapped around *bolAp2*. The last protection zone corresponds only to a 3 bps sequence and it may not be significant. Even though H-NS was considered a non-sequence specific binding protein, recent studies defined that this global regulatory protein interacts with AT-rich regions commonly found in bacterial gene promoters [21]. A consensus region, and a consensus structure (DNA curvature) for protein–DNA interaction has also been identified [21,24,25]. In these experiments, the regions of interaction were confirmed to be AT-rich, matching the characteristics of the high affinity H-NS interaction zones and, all the main interaction zones identified share a partial similarity with the 10 bp described consensus (TCGTTAAATT) [21] (see Fig. 4). Altogether, our results support H-NS ability to bind simultaneously to several sites within the entire regulatory region of *bolA*, and form higher order structures originating a repressive nucleoprotein complex that modulates the activity of *bolAp1* and *bolAp2*.

In this report we showed that the pleiotropic histone-like protein H-NS is a new transcription regulator of *bolA* and we have characterized its mode of action. We demonstrated that H-NS is directly repressing *bolA* expression by binding to different locations along its entire promoter regions. Four major interaction zones were identified encompassing both *bolAp2* and *bolAp1* promoters. Moreover, the binding sites are confined to a curved DNA region, acknowledged to be the H-NS preferred consensus structure.

BolA has been shown to be a pleiotropic protein that affects several cellular functions. It has been described as a transcription factor, as well as a morphogene [2,4,26]. It was also shown to be important for cell survival [7]. In this context, a fine tuned regulation of this gene may be essential for the cell. This work adds a new regulator, H-NS, to the already complex network of BolA modulators. H-NS is known to be involved in flagella biosynthesis [27]. Additionally, in *E. coli*, bacterial motility influence biofilms architecture [28]. We have previously shown that *bolA* can induce biofilm formation [6], therefore H-NS and BolA may be involved in

the molecular mechanisms that control the link between motility and biofilm development.

Acknowledgments

The authors would like to thank Ana Barbas for the technical support in the performed EMSA assays and Patrick Freire for the critical reading of the manuscript. This work was supported by Fundação para a Ciência e Tecnologia (FCT) (Portugal). Ricardo Moreira was supported by a Doctoral fellowship from FCT and Clémentine Dressaire and Susana Domingues were recipients of Postdoctoral fellowships, also from FCT.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.06.084.

References

[1] J.M. Santos, P. Freire, M. Vicente, C.M. Arraiano, The stationary-phase morphogene bolA from *Escherichia coli* is induced by stress during early stages of growth, Mol. Microbiol. 32 (1999) 789–798.
[2] M. Aldea, C. Hernandez-Chico, A.G.d.l. Campa, S.R. Kushner, M. Vicente, Identification, cloning, and expression of bolA, an ftsZ-dependent morphogene of *Escherichia coli*, J. Bacteriol. 170 (1988) 5169–5176.
[3] M. Aldea, T. Garrido, C. Hernandez-Chico, M. Vicente, S.R. Kushner, Induction of a growth-phase-dependent promoter triggers transcription of bolA, an *Escherichia coli* morphogene, EMBO J. 8 (1989) 3923–3931.
[4] P. Freire, R. Neves Moreira, C.M. Arraiano, BolA inhibits cell elongation and regulates MreB expression levels, J. Mol. Biol. 385 (2009) 1345–1351.
[5] J.M. Santos, M. Lobo, A.P. Matos, M.A. De Pedro, C.M. Arraiano, The gene bolA regulates dacA (PBP5), dacC (PBP6) and ampC (AmpC), promoting normal morphology in *Escherichia coli*, Mol. Microbiol. 45 (2002) 1729–1740.
[6] H.L. Vieira, P. Freire, C.M. Arraiano, Effect of *Escherichia coli* morphogene bolA on biofilms, Appl. Environ. Microbiol. 70 (2004) 5682–5684.
[7] P. Freire, H.L. Vieira, A.R. Furtado, M.A. de Pedro, C.M. Arraiano, Effect of the morphogene bolA on the permeability of the *Escherichia coli* outer membrane, FEMS Microbiol. Lett. 260 (2006) 106–111.
[8] L.H. Nguyen, R.R. Burgess, Comparative analysis of the interactions of *Escherichia coli* sigma S and sigma 70 RNA polymerase holoenzyme with the stationary-phase-specific bolAp1 promoter, Biochemistry 36 (1997) 1748–1754.
[9] D.R. Gentry, V.J. Hernandez, L.H. Nguyen, D.B. Jensen, M. Cashel, Synthesis of the stationary-phase sigma-factor sigma(S) is positively regulated by Ppgpp, J. Bacteriol. 175 (1993) 7982–7989.
[10] R. Lange, R. Hengge-Aronis, Growth phase-regulated expression of bolA and morphology of stationary-phase *Escherichia coli* cells are controlled by the novel sigma factor sigma S, J. Bacteriol. 173 (1991) 4474–4481.
[11] K. Yamamoto, R. Nagura, H. Tanabe, N. Fujita, A. Ishihama, R. Utsumi, Negative regulation of the bolA1p of *Escherichia coli* K-12 by the transcription factor OmpR for osmolarity response genes, FEMS Microbiol. Lett. 186 (2000) 257–262.
[12] J.M. Santos, P. Freire, F.S. Mesquita, F. Mika, R. Hengge, C.M. Arraiano, Poly(A)-polymerase I links transcription with mRNA degradation via sigmaS proteolysis, Mol. Microbiol. 60 (2006) 177–188.
[13] C.M. Arraiano, J.M. Andrade, S. Domingues, I.B. Guinote, M. Malecki, R.G. Matos, R.N. Moreira, V. Pobre, F.P. Reis, M. Saramago, I.J. Silva, S.C. Viegas, The critical role of RNA processing and degradation in the control of gene expression, FEMS Microbiol. Rev. 34 (2010) 883–923.
[14] P. Freire, J.D. Amaral, J.M. Santos, C.M. Arraiano, Adaptation to carbon starvation: RNase III ensures normal expression levels of bolA1p mRNA and sigma(S), Biochimie 88 (2006) 341–346.
[15] M. Barth, C. Marschall, A. Muffler, D. Fischer, R. Hengge-Aronis, Role for the histone-like protein H-NS in growth phase-dependent and osmotic regulation of sigma S and many sigma S-dependent genes in *Escherichia coli*, J. Bacteriol. 177 (1995) 3455–3464.
[16] C.J. Dorman, H-NS: a universal regulator for a dynamic genome, Nat. Rev. Microbiol. 2 (2004) 391–400.
[17] T. Baba, T. Ara, M. Hasegawa, Y. Takai, Y. Okumura, M. Baba, K.A. Datsenko, M. Tomita, B.L. Wanner, H. Mori, Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection, Mol. Syst. Biol. 2 (2006).
[18] J. Sambrook, D. Russell, Molecular Cloning, A Laboratory Manual, third ed., Cold Spring Harbor Laboratory, NY, 2001.
[19] T. Moss, DNA–Protein Interactions, Principles and Protocols (Methods in Molecular Biology), Humana Press, 2001.
[20] M. Mujacic, F. Baneyx, Regulation of *Escherichia coli* hchA, a stress-inducible gene encoding molecular chaperone Hsp31, Mol. Microbiol. 60 (2006) 1576–1589.
[21] B. Lang, N. Blot, E. Bouffartigues, M. Buckle, M. Geertz, C.O. Gualerzi, R. Mavathur, G. Muskhelishvili, C.L. Pon, S. Rimsky, S. Stella, M.M. Babu, A. Travers, High-affinity DNA binding sites for H-NS provide a molecular basis for selective silencing within proteobacterial genomes, Nucleic Acids Res. 35 (2007) 6330–6337.
[22] C.A. White-Ziegler, T.R. Davis, Genome-wide identification of H-NS-controlled, temperature-regulated genes in *Escherichia coli* K-12, J. Bacteriol. 191 (2009) 1106–1110.
[23] F.C. Fang, S. Rimsky, New insights into transcriptional regulation by H-NS, Curr. Opin. Microbiol. 11 (2008) 113–120.
[24] H. Yamada, S. Muramatsu, T. Mizuno, An *Escherichia coli* protein that preferentially binds to sharply curved DNA, J. Biochem. 108 (1990) 420–425.
[25] M. Sette, R. Spurio, E. Trotta, C. Brandizi, A. Brandi, C.L. Pon, G. Barbato, R. Boelens, C.O. Gualerzi, Sequence-specific recognition of DNA by the C-terminal domain of nucleoid-associated protein H-NS, J. Biol. Chem. 284 (2009) 30453–30462.
[26] I.B. Guinote, R. Matos, P. Freire, C. Arraiano, BolA affects cell growth, and binds to the promoters of penicillin-binding proteins 5 and 6 and regulates their expression, J. Microbiol. Biotechnol. 21 (2011) 243–251.
[27] P. Bertin, E. Terao, E.H. Lee, P. Lejeune, C. Colson, A. Danchin, E. Collatz, The H-NS protein is involved in the biogenesis of flagella in *Escherichia coli*, J. Bacteriol. 176 (1994) 5537–5540.
[28] T.K. Wood, A.F. Gonzalez Barrios, M. Herzberg, J. Lee, Motility influences biofilm architecture in *Escherichia coli*, Appl. Microbiol. Biotechnol. 72 (2006) 361–367.

Characterization of the BolA homologue IbaG: a new gene involved in acid resistance

Inês Batista Guinote[1,2], Ricardo Neves Moreira[1], Patrick Freire[1,2] and Cecília Maria Arraiano[1] ✉
[1]Instituto de Tecnologia Química e Biológica/Universidade Nova de Lisboa, Apartado 127, 2781-901 Oeiras, Portugal
[2]Present address: Laboratório Nacional de Investigação Veterinária - INRB, Estrada de Benfica 701, 1549-011 Lisboa, Portugal

Abstract

The BolA protein homologues are widely distributed in nature. In this report, we have studied for the first time YrbA, the only BolA homologue present in *E. coli*, which we have renamed as *ibaG*. We have constructed single and multiple *ibaG* mutants, and overexpression plasmid in order to characterize this gene. IbaG phenotypes are different from the *bolA* associated round morphologies or growth profiles. Interestingly, the *ibaG* and *bolA* single and double deletion mutants grow faster and have higher viabilities, in rich medium, while the overexpression strains are significantly growth impaired. However the mutant strains have lower viabilities than the wild type in late stationary phase, indicating that both *bolA* and *ibaG* are important for survival in difficult growth conditions. In this work, *ibaG* was also demonstrated not to interact with DNA fragments differing from the BolA transcriptional factor, but to change its mRNA expression pattern in response to acidic stress. *IbaG* may represent a new gene involved in cell resistance against acid stress.

Keywords: *yrbA*, BolA, acid stress

*Corresponding author: Fax: +351 214469549
E-mail: cecilia@itqb.unl.pt (CM. Arraiano)

Introduction

The BolA protein homologues are widely distributed in nature with exception of Gram-positive bacteria. Moreover some organisms have several genome copies. Although their cellular role is still an open field to research, these proteins seem to be involved in protection from stress and cell proliferation or cell-cycle regulation [15]. The overall topology of a mouse BolA-like protein is similar to the class II KH fold [1]. Interestingly, all the conserved residues in the BolA-like proteins are assembled in one side of the protein [14]. *E. coli* BolA acknowledged amongst several transcriptional factors [20]. It exhibits an helix-turn-helix motif that may correspond to a DNA-binding domain, through which it can eventually interact and transcriptionally regulate different genes [1]. In agreement, BolA was shown to repress the actin-like *E. coli* protein MreB [9] and to induce the DD-carboxypeptidases PBP5 and PBP6 [12, 29]. When *bolA* is overexpressed, the cells reduce size and present a spherical morphology [3, 28, 29]. The rod to sphere shape modulation occurs from exponentially growing to stationary phase cells, in a FtsZ dependent manner [3, 16]. Moreover, *bolA* overexpression induces biofilm formation [30], and alters the outer membrane

Table 1. Bacterial Strains and Plasmids

Strains	Description	Reference or source
BL21(DE3)		Novagen commercial strain
BT340	DH5α+pCP20 (thermosensitive plasmid expressing recA, to RFFP excise the antibiotic cassette inserted in the genome)	(Cherepanov and Wackernagel, 1995)
CMA10	MG1693 + pMAK580 (strain with plasmid overexpressing bolA)	Santos et al., 1999
CMA50	BL21 (DE3) + pPFA02 (plasmid over-expressing (His)6–BolA)	(Freire et al., 2009)
CMA63	BL21+p363 (plasmid encoding for GFP after Multi Cloning Site to evaluate transcription levels of the genes after the promoters inserted)	(Freire et al., 2009; Miksch et al., 2005)
CMA83	MG1693 + pBr325 (Gibco Brl commercial plasmid)	(this study)
CMA84	MG1693 + pBGA01 (plasmid overexpressing *yrbA*[*ibaG*])	(this study)
CMA85	MG1693 Δ*ibaG*::Kan^r (*yrbA*[*ibaG*] deletion mutant _ Keio derived)	(this study)
CMA86	MG1693 Δ*bolA*::Kan^r (*bolA* deletion mutant (Keio derived)	(this study)
CMA87	MG1693 Δ*bolA* (*bolA* deletion mutant without the Kanamycin resistance cassette)	(this study)
CMA88	MG1693 Δ*bolA*Δ*ibaG*::Kan^r (double *bolA* & *yrbA*[*ibaG*] deletion mutant)	(this study)
CMA89	MG1693+pRMA02 (plasmid expressing GFP downstream the *yrbA*(*ibaG*) promoter)	(this study)
CMA90	MG1693Δ*bolA*::Kan^r+pRMA02	(this study)
CMA91	BL21 (DE3) + pPFA02+pRMA02 (CMA50 with plasmid expressing GFP downstream the *yrbA*(*ibaG*) promoter)	(this study)
JW3157	MG1655Δ*ibaG*::Kan^r (Keio collection mutant for *yrbA*[*ibaG*])	(Baba et al., 2006)
JW5060	MG1655Δ*bolA*::Kan^r (Keio collection mutant for *bolA*)	(Baba et al., 2006)
MG1693	thyA715 (background strain)	Bachmann and Low, 1980

properties namely accessibility and sensitivity towards detergents and antibiotics [10]. Those biochemical and physiological alterations may depend on the role of BolA regulation over inner membrane proteins [1].

The *bolA* gene is preceded by two promoters: *bolA2p* and *bolA1p*. The upstream promoter *bolA2p* is weak, constitutive, and dependent on σ^{70}. The *bolA1p* expression is driven by σ^S and is a gearbox promoter, showing an activity inversely dependent on the growth rate [1-3]. BolA transcription increases substantially in the transition to stationary phase [1]. The *bolA1p* promoter is also induced in exponential phase in response to several stresses (heat, acid, oxidative, osmotic and glucose depletion) [3, 28]. At the post-transcriptional level, BolA is indirectly modulated by Ribonuclease III that increases *rpoS* mRNA and σ^S protein levels [8, 26]. Polyadenylation also reduces σ^S proteolysis, and consequently affects *bolA* [27]. Since this gene is strongly expressed under stress and quite promptly repressed when growth conditions become favourable, *bolA* is suggested to be involved in promoting general resistance mechanisms. In agreement, the *bolA* yeast homologue is a UV-inducible gene which accelerates spore formation, decreases proliferation rate, enhances cell size, confers UV resistance and is eventually

responsible for the control of cell division, especially on resumption from cell cycle arrest [15].

Given the importance of BolA in several challenging environments *in silico* analysis (protein BLAST) was performed and a BolA homologue protein (YrbA) was found in *E. coli*. YrbA has 23% of amino acid overall identity, 58% of similarity at the BolA/YrbA domain, and over 70% of the amino acid residues of both BolA and YrbA proteins can be aligned. Similarly to BolA, YrbA has a helix-turn-helix motif, usually responsible for protein-DNA interaction.

In this work we have constructed a single isogenic *yrbA* mutant and double *bolA*/*yrbA* mutant to evaluate in reference to the *bolA* mutant and wild type strain. YrbA overexpression and characterization upon several growth conditions was also performed, and the results have shown that this gene is responsive to acid stress, therefore we have proposed a new name for *yrbA:* **i**nfluenced **b**y **a**cid **g**ene, *ibaG*.

Materials and Methods

Bacterial Strains and Plasmids

The strains and plasmids used in this study are described in Table 1. MG1693 chromosomal DNA was used as template for *ibaG* and surrounding regions PCR using *Pfu* DNA polymerase (Fermentas) and the primer pair pCLON1/pCLON2 (Table S1). DNA template was prepared using the genomic DNA purification kit from PUREGENE™DNA Cell & Tissue Kit Purification System from Gentra Systems. Both pBR325 and the portion of genome amplified contained the *Aat*I and *Pts*I restriction sites that were separately digested. The 5319 bp plasmid fragment and the PCR fragment digestion were purified with the illustra™ GFX™ PCR DNA and Gel Band Purification Kit from GE Healthcare. Overnight ligation was performed with T4 DNA ligase (Roche) to produce the pBGA01 plasmid.

The *bolA* and *ibaG* deletions were transferred from the Keio collection deletant strains to the MG1693 strain. The Keio collection deletant strains were kindly provided by Keio University [4]. All deletion constructs from Keio were performed inserting a kanamycin resistance cassette while removing the genes with exception of the first and last few base pairs. This prevented frameshift and downstream genes expression to be affected in the case of operon co-expressed genes. In this case, the *ibaG* (previously designated *yrbA*) gene was removed maintaining the upstream promoter region and the downstream essential gene expression unperturbed [4]. Gene transfer was achieved by P1-mediated transduction according to the method previously described [22]. For construction of the double deletion mutant the kanamycin resistance cassette (introduced to delete the *bolA* gene) was eliminated before the second transduction. The FRT (FLP recombination target) sites flanking the antibiotic resistance cassette were eliminated by recombination by the FLP recombinase encoded in the pCP20 plasmid that was transformed and then temperature cured, following the published protocol [7].

The plasmid pRMA2 was constructed encoding the *gfp* gene (for green fluorescent protein) under the

control of *ibaG* promoters, using the vector p363 [21]. To do so, the *ibaG* promoter was PCR amplified using the primers yrFw and yrRev (see Table S1). The result fragment was digested with *ClaI* (Fermentas) and ligated to the p363 fragment digested with the same restriction enzyme. When necessary, strains were transformed with plasmid pCP20 (commercial plasmid), pBR325 (commercial plasmid), pBGA01 (this study), pMAK580 [3], or pRMA02 (this study). Transformations were carried out as previously described [25]. All plasmids and deletion mutants were confirmed by DNA sequencing at STAB Vida, Portugal.

Media, growth conditions, and viability evaluation

Luria broth (LB), M9, and Luria agar (LA) compositions were prepared as previously described [22]. When required, the media were supplemented with 0.4 mM thymine, 50 mg/ml chloramphenicol, 50 mg/ml kanamycin, 0.04% glucose (w/v), (all from Sigma) and 1 mM IPTG (from Apollo Scientifics). For acid and osmotic challenge experiments, strains were grown in LB. Overnight grown cultures were diluted to an OD_{620nm} of 0.08 and allowed to grow until an OD of about 0.5. At such moment all cultures were centrifuged at 5500 g for 15 minutes at 4 ºC, to change media into a buffered LB at different pH or ionic strengths. Tests were performed at pH3, 4, and 5 in LB buffered with sodium citrate and LB plus citric acid, or LB with 87.5 mM, 175 mM, and 350 mM NaCl, according to previous description [17]. Optical densities were measured in an Amersham Biosciences Ultrospec®500/1100pro spectrophotometer at 620nm, using 10 mm light path cuvettes. The ODs were determined according to the Lambert-Beer law's limits of direct proportionality between OD and sample concentration (dilutions were made in LB so that density values would be read between 0.02 and 0.6); the phases of growth analyzed were determined according to the growth curves. Batch cultures were either launched from 16h overnight inoculi at 30ºC (for mild growth) or 37 ºC and 100 rpm, which were diluted to an optical density of 0.08 measured at 620 nm (OD_{620nm}). Cultures were grown aerobically at 37 ºC and 120 rpm. For nutritional stress evaluation cells were grown in M9 supplemented with glucose until an OD_{620nm} of 0.35 - corresponding to exponential phase (M9 Exp), washed twice in M9 without glucose and resumed growth in the same glucose depleted media for 60 minutes – corresponding to starvation (Starv 1h). After one hour of glucose starvation cultures were re-added with glucose for some additional 15 minutes – corresponding to reversion (Rev 15 min). For evaluation of viability, the samples were processed in LB serial dilutions, and plated in LA. The number of colony forming units (cfu) was counted and viability was determined according to the equation: Number of dividing cells per ml = cfu x $10^{dilution}$ x 1000/100.

Microscope preparations

To observe the effect of IbaG (YrbA) on cells morphology, planktonic cells were harvested from cultures growing in LB, at the time points corresponding to log, early exponential, late

exponential, early stationary and late stationary phases, or from cultures growing in M9 at exponential phase (M9 Exp), after one hour of starvation (Starv 1h), and after 15 minutes of reversion (Rev 15 min). Cells were fixed with 0.75% (v/v) formaldehyde and stored at 4 ºC. For the Differential Interference Contrast (DIC) microscopy photographs, 20 μl of the samples were observed in slides coated with a thin 1.5% (w/v) agarose film, and enclosed with nr.1 cover glass. Images were obtained using a DMRA microscope (Leica) under Nomarski optics coupled to a CCD camera, with Metamorph software.

Overexpression and purification of BolA protein

BolA overexpression using the pPFA02 plasmid and sequential purification was performed according to previous description [12]. The plasmid used for expression of BolA was a pET28a derived pPFA02 [9] transformed into a Novagen *E. coli* BL21 (DE3) strain (Table 1). Purification of BolA was performed by histidine affinity chromatography using His Trap Chelating HP columns (GE Healthcare) and AKTA fast protein liquid chromatography system (GE Healthcare). Proteins were eluted with a continuous imidazol gradient (until 100 mM) and buffer was exchanged to 20mM Sodium Phosphate pH7.4 and 50mM NaCl buffer. Protein concentration was determined by spectrophotometry using a Nanodrop device and measuring the OD at 280 nm. 10μl of purified protein fractions were applied to a 15% SDS-PAGE and visualized by Coomassie blue staining to assess protein purity (data not shown).

Surface Plasmon Resonance (SPR) Analysis

The SPR analysis was performed in a BIACORE 2000 instrument. Purified BolA protein was immobilized in a CM5 sensor chip by amine coupling immobilization method according to the manufacturer's instructions (GE Healthcare). The same immobilization procedure was performed with the same molarity of BSA control protein in a reference flow cell, used to correct for refractive index changes and non-specific binding [12]. The *ibaG* promoter and open reading frame (ORF) were amplified by PCR using primers yrFw/YrRev and 3/5 primers, respectively (see Table S1). To create a fragment containing the ORF exclusively, the second PCR product was digested with NcoI, and purified with the Nucleic Acid and Protein Purification kit: NucleoSpin®Extract II, from Macherey-Nagel. The promoter sequence of the *mreBCD* operon was used as a positive control, and the *bolA* open reading frame (ORF) DNA encoding fragment as a negative control, as previously described [9]. The assays were run at 25 ºC in 20 mM Sodium Phosphate pH7.4, 1 mM dithiothreitol, and 500mM NaCl buffer as previously described [12]. Equilibrium constants were determined using the BIA Evaluation 3.0 software package, according to the fitting model 1:1 Langmuir Binding, and $\chi 2$ statistics were used to evaluate the fitness of the model to the data.

RNA extraction and probe preparation

Culture samples were taken at the desired time points along the growth curve – OD_{620nm}= 0.4, 1.7, and 5 - or after imposition of osmotic or acidic stresses: 350 mM

NaCl [23] or 30% HCl, lowering the pH at 7.2 to 4.4 [5] both at time 0 and 60 minutes. Total RNA was extracted as described [26]. In all experiments 1 µl of the RNA samples was quantified by UV spectrometry with NANODROP and integrity was verified. The probes used for *bolA* and *ibaG*, in Northern blot experiments spanned the entire transcriptional units and were obtained by PCR, using Taq polymerase (Fermentas) and respectively the primers P2/X9 and 3/5, in the case of DNA probes, or P2/X9_T7 and 3/5_T7, in the case of RNA probes (see Table S1). 3 µl of pUC Mix Marker 8 (Fermentas) were labeled with [γ-^{32}P] ATP using PNK and 100 times diluted into the RNA loading buffer. 7.5 µl of labeled pUC 8 were run simultaneously with the samples to determine their molecular weight. All radioactive labels were cleaned in G-50 columns from GE healthcare.

Reverse Transcription PCRs (RT-PCR)

Reverse transcription-PCRs (RT-PCRs) were carried out with 50 ng of total RNA, with the OneStep RT-PCR kit (Qiagen), according to the supplier's instructions, using the oligonucleotides RNM017 and 5 (see Table S1). As an independent control, the 16S rRNA-specific primers 16sF and 16sR were used. Prior to RT-PCR, all RNA samples were treated with TURBO DNA-free™ Kit (Ambion). Control experiments, run in the absence of reverse transcriptase reactions, yielded no product.

Northern blot and hybridization

Samples containing 15 µg of total RNA were dissolved in 90% formamide, 0.01 M EDTA pH7.0, 1 mg/ml xylene cyanol, 1 mg/ml bromophenol blue buffer [25], heated for 5 min at 100ºC for denaturation, and incubated for 10 min on ice. Total RNA samples were electrophoresis run on a 6% denaturing polyacrylamide gel and transferred to a nylon membrane (Amersham Hybond™-N$^+$ nitrocellulose) according to the procedure described by Fitzwater *et al*. (1987). The RNA was then fixed to the membrane by UV light and hybridized with the PCR probe radiolabelled with [α-32P]-dCTP, using the Multiprime DNA labelling system from Amersham or with [α-^{32}P]-rUTP, using the Promega labeling system for riboprobes. Probe hybridization with PerfectHyb™Plus Hybridization Buffer 1x, was carried out at 42ºC for DNA probes and 68ºC for RNA probes. Amersham Hybond™-N$^+$ nitrocellulose membranes optimized for nucleic acid transfer from GE Healthcare were hybridized and washed as described by Sambrook *et al*. (1989). The results were visualized using the PhosphoImager System from Molecular Dynamics.

Transcription evaluation using GFP as reporter

Transcription evaluation was analyzed with *gfp* as reporter gene using the p363-derived vector [21] pRMA02 (see *Bacterial strains, plasmids and genetic manipulations*). BL21 + pPFA02 + pRMA02 was grown at 37ºC, 120 rpm until OD_{620nm} = 0.5 when the culture was split in two. Half the culture was added with 1mM IPTG (to induce *bolA* expression) and the other with 0.04% glucose (to repress *bolA* expression). In a parallel experiment MG1693 and the isogenic *bolA* deletant were grown until OD 1.7 (the mid

exponential transcripts evaluation time point). Total protein was extracted using Bugbuster reagent (Novagen) and GFP fluorescence was quantified in a Varian-Eclipse Spectrofluorimeter. SDS-PAGE gels and Western-blots were performed as described before [9]. Results were normalized and are shown in percentage (%) as ratio of fluorescence / EF-Tu quantified in the Western-blots. Final data represents the average plus standard deviation of fluorescence per cell, from at least three independent experiments.

Primer extension analysis

Total RNA was extracted as described above. The primer PExtYrbA was end-labelled using T4 polynucleotide kinase (Fermentas) and [γ-^{32}P] ATP. Unincorporated [γ-32P] ATP was discarded using a MicroSpinTM G-25 Column (GE Healthcare). A total of 2 pmol of primer was annealed to 10 mg of RNA and cDNA was synthesized using 200U of Superscript III RT from Invitrogen. M13 sequencing reaction was performed with Sequenase Version 2.0 sequencing kit according to the instructions manual. The primer extension products were separated in parallel with the M13 sequencing reaction on a 6% polyacrylamide sequencing gel containing 8M urea. The gel was exposed and signals were visualized in a PhosphorImager (Storm Gel and Blot Imaging system, Amersham Bioscience).

Results & Discussion

IbaG is the only BolA homologue in E. coli

NCBI public resources were used to search for potential homologues of *bolA* and an uncharacterized gene was found to have a strong protein similarity with BolA, particularly considering the shared

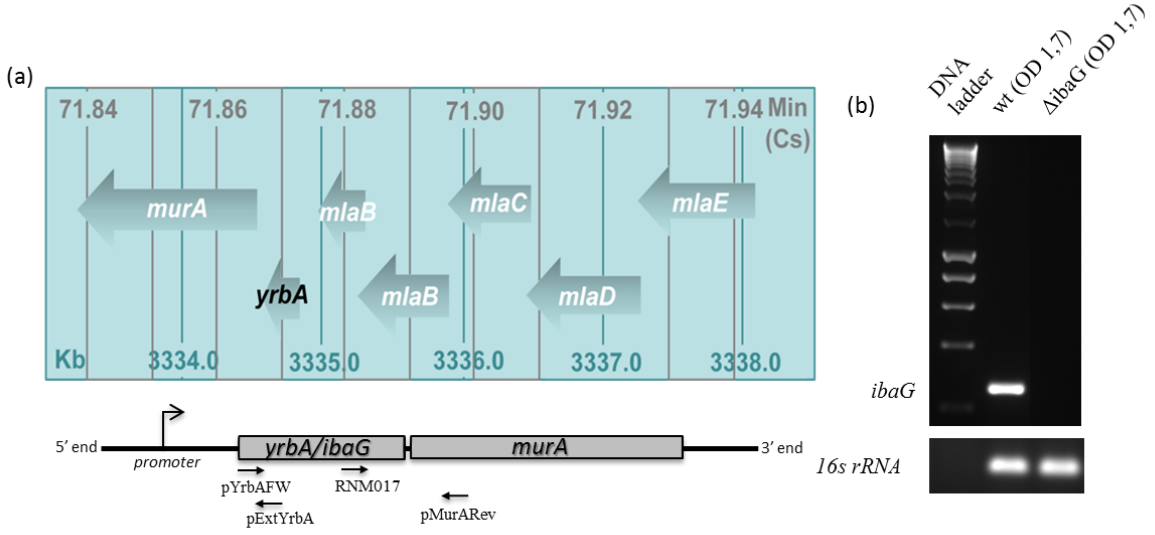

Figure 1a. *ibaG* and adjacent *Escherichia coli* genes (adapted from Ecogene public server); below are represented the primers used to evaluate *ibaG* transcript levels. 1b. *ibaG* is co-transcribed with *murA*. RT-PCR amplification of *ibaG* transcript from total RNA of *E. coli* in exponential phase (1 hour after OD 0.5) and mutant *ΔibaG* as a negative control. Control experiments performed with 16s rRNA specific primers indicated that there were no significant differences in the amounts of RNA in each sample.

bolA/*yrbA* domain. *ibaG* gene is at the 71.87 minutes of the *E. coli* genome, downstream of an operon of five genes co-directionally expressed (Fig. 1A). Nevertheless *ibaG* is not predicted to be co-expressed with the upstream operon, but from its own σ^{70} single promoter region as evaluated by the REGULON DB 6.7: Gene Form [11]. The upstream genes *mlaBCDEF* (plus *mlaA*) compose the Mla pathway, an ABC transport system whose function seems to prevent phospholipidic accumulation in the outer leaflet of Gram-negative bacteria, thus contributing to the preservation of the outer membrane lipid asymmetry [18]. The essential *murA* gene, is encoded downstream to *ibaG*. This vital gene encodes for the UDP-N-acetylglucosamine enolpyruvyl transferase, which synthesizes peptidoglycan precursors after N-acetylglucosamine acid and phosphoenoylpyruvate [6, 13, 19]. All of these seven proteins are predicted to occur or be function related to the outer membrane and are either essential or significantly affect the ability to resist against external injuries. The genes that occur in the proximity of *ibaG*, as well as the sequence homology that this gene has with *bolA*, determined the importance of its evaluation.

In order to characterize the *ibaG* transcriptional unit, Neural Network Promoter Prediction [24] software was also used to search putative promoters upstream of *ibaG* ORF. One putative promoter region with a 0.7 score was found matching the ATG start codon already described in different databases. Primer extension reaction was performed in order to map the transcription start site of *ibaG* mRNA. The +1

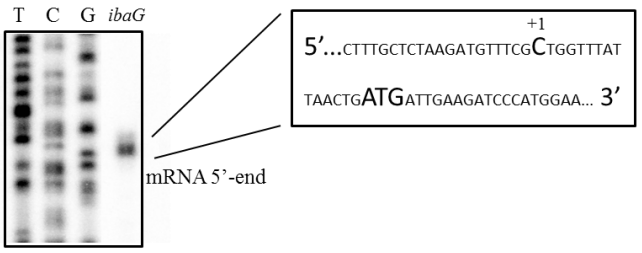

Figure 2. 5'-end mapping of *ibaG* transcriptional unit. Reverse transcription with gene specific primer was performed to extend the 5' region of *ibaG* mRNA. The first nucleotide of the transcript (+1) was identified with aid of an M13 sequencing reaction and is represented in the above sequence.

nucleotide was determined and identified 15 bp before the start codon of the *ibaG* mRNA (Fig 2). For the transcription end, a putative terminator is described in REGULON DB 6.7: Gene Form[11]. Interestingly, this transcription terminator is overlapping with the downstream *murA* gene. As a result, Reverse Transcription PCR (RT-PCR) was used to confirm if *ibaG* is present exclusively as a single transcriptional unit or if it can be co-transcribed with the *murA* gene. We observed that *ibaG* is in fact transcribed in an operon transcriptional unit with the *murA* essential gene (Fig. 1B). This relates *ibaG* with the metabolism of the murein precursors and might suggest a role for this gene in that process.

ibaG is not an essential gene and both single and double bolA/ibaG deletions improve E. coli growth

Similarly to what happens with *bolA*, the single deletion of *ibaG* is not lethal [4]. Moreover, if *ibaG* could complement any essential *bolA* functions or vice-versa, the double deletion *ΔbolAΔibaG* would be expected to be lethal. However, the double mutant strain is also viable. Growth, viability, and

morphological analysis were performed to check the phenotypical effects due to the absence of these proteins. In the optimal growth conditions used, and contrarily to what had been anticipated, the deletion mutants grew similarly or better than the wild type (wt) strain MG1693. Both Δ*ibaG* and Δ*bolA*Δ*ibaG* deletion mutants grew about 20% times faster (evaluated by the exponential phase rate of growth) and reach higher OD than the wild type (Fig. 3A). These results were confirmed by the number of colony forming units obtained for the lag, early exponential, mid exponential, and late exponential/early stationary phases of growth (Fig. 3B). The wt MG1693 strain forms about half or even less colonies than any of the deletion mutants, with the exception of late stationary phase. In the beginning of the growth curve, and until early stationary phase, all cultures reveal a viability increment. However, there is a transitory decrease in the viabilities of the Δ*bolA* strain at mid exponential phase, perhaps when the stimulus for *bolA* expression is occurring. It is also possible to distinguish the deletion strains based on the colony forming units (cfu). The cfu are increasingly higher from the Δ*bolA* to the Δ*ibaG*. Moreover, the Δ*bolA*Δ*ibaG* cell counts are higher even compared to the single mutants. The wt viabilities are quite stable along the entire growth curve. Contrasting, the deletion strains largely reduce their cell counts in late stationary phase, strengthening the idea that these genes may be important for survival in difficult growth conditions. Given that all deletion strains significantly increase their cell counts until late stationary phase, the survival or tolerance that these genes provide at such phase seems to occur at the expense of previous growth, as occurs in several other stress response genes. It seems to reflect the trade-off between growth and resistance related to the σ^{70} *versus* σ^{S} expression pattern. Eventually, the decrease in growth potential due to the presence of both genes in the wild type

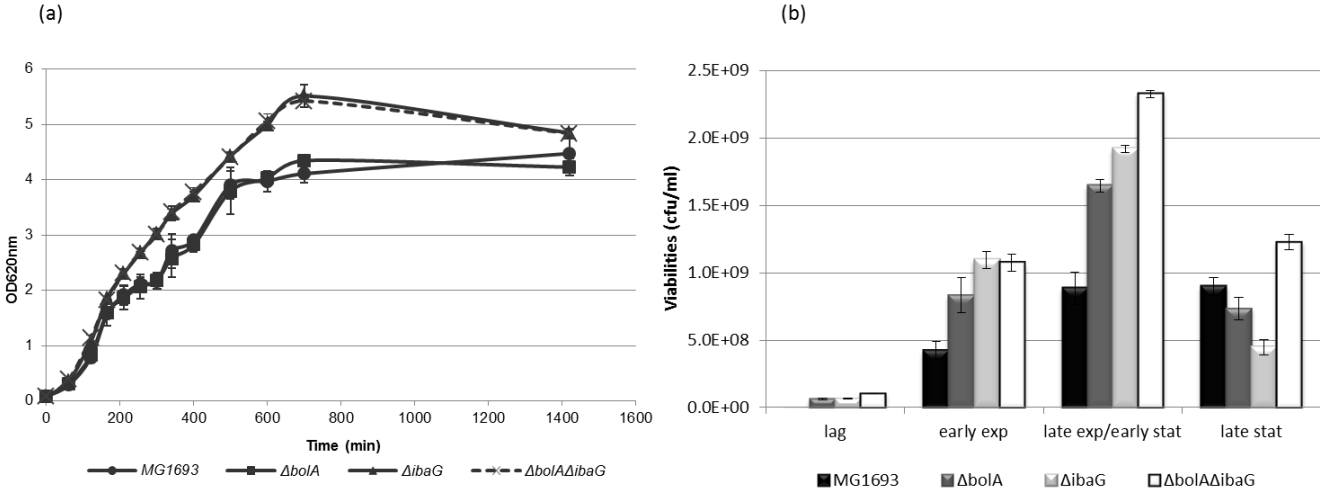

Figure 3. Growth curves and viabilities depending on the absence of *bolA, ibaG*, or both genes. (a) MG1693, MG1693Δ*bolA*, MG1693Δ*ibaG* and MG1693Δ*bolA*Δ*ibaG* optical density measurements at 620 nm, for determination of growth curves in LB medium at 37ºC and 120 rpm. (b) Viability evaluated by number of colony forming units in properly supplemented LA plates for the same strains and conditions.

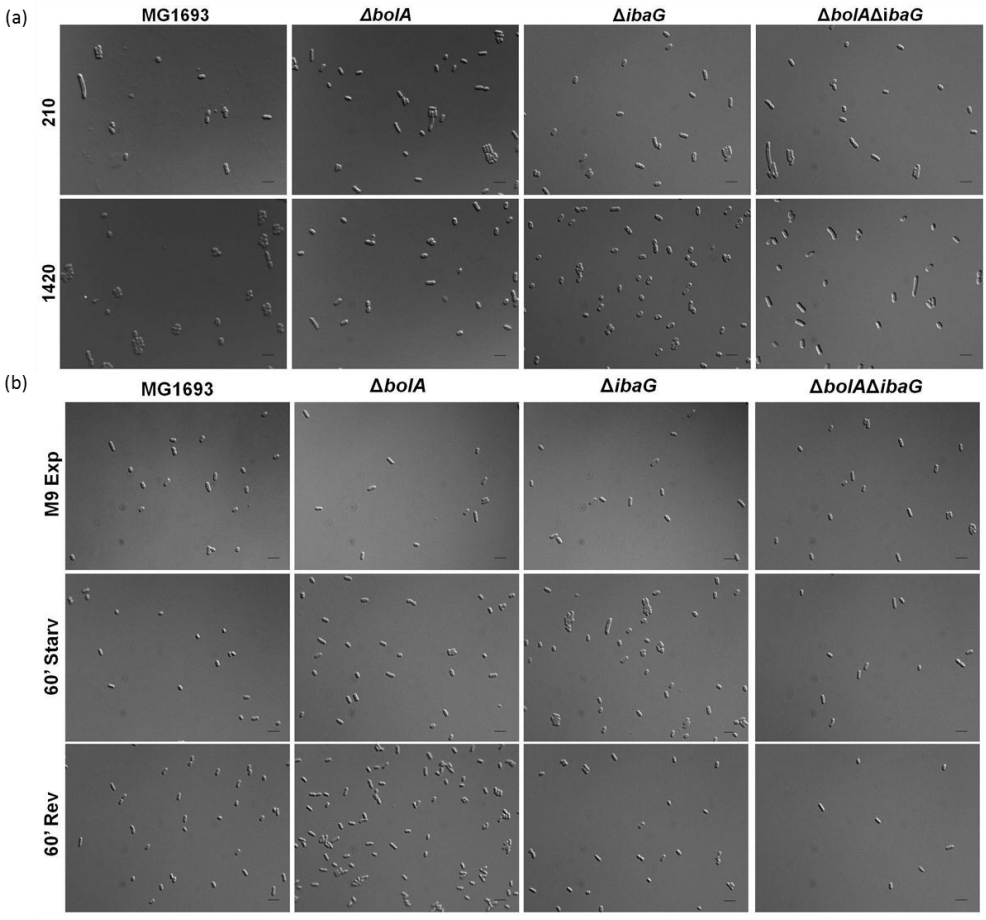

Figure 4. Differential Interference Contrast microscopy photographs, obtained using a DMRA microscope (Leica) for the strains MG1693, MG1693Δ*bolA*, MG1693Δ*ibaG*, and MG1693Δ*bolA*Δ*ibaG*. (a) Evaluation at time points 210 and 1420 min of the LB growth; (b) Evaluation at exponential phase, one hour after induced starvation, and fifteen minutes after reversion, in M9 minimal medium. The dark bar represents 5 µm.

exponential phase may reduce the exhaustion of important resources or prevent some catabolites from being produced and released to the media, thus favoring the population maintenance in late stationary phase (Fig. 3B).

Morphology assessment was made for wt and all deletion strains at the same time points where viability was evaluated. All strains evolved similarly (Fig. 4A). Given that *bolA* and *ibaG* may be involved in the cell protection against stresses, morphologies were also analyzed in poor or nutritional stress conditions: M9 minimal media growth, one hour of glucose starvation at exponential phase and 15 minutes of nutritional stress reversion by re-addition of glucose. The results did not diverge according to strains (Fig. 4B). Not only the proteins, but also the growth behavior of the deletion strains was very similar.

Morphologies are not altered by an increase in ibaG copy number

The deletion of *bolA* does not significantly affect the morphology of the cells during growth in LB. Nevertheless, when it is overexpressed, *bolA* does change the cells shape from rod to spheres [3]. To further pursue the characterization of *ibaG* we have cloned it into a pBR325 derived plasmid preceded by the respective promoter (pBGA01). Growth curves, viabilities assessment and morphological studies were performed. The MG1693 strain transformed with pBR325 was used as an additional control or wt strain when evaluating the effects of the plasmids pMAK580 (overexpressing *bolA*) and pBGA01 (overexpressing *yrbA*). The growth of MG1693 and MG1693 transformed with pBR325 did not depend on the

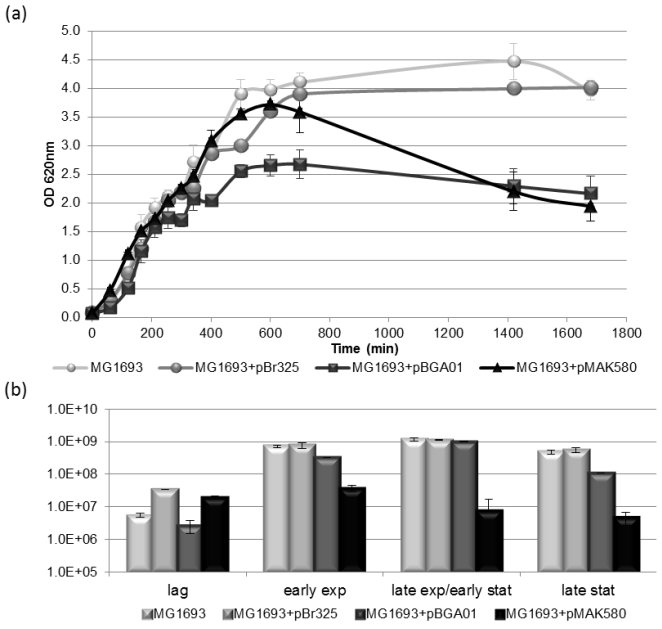

Figure 5. Growth curves and viabilities induced by the excess of *bolA*, and *ibaG*, or the control plasmid. (a) MG1693, MG1693+pMAK580, MG1693+pBGA01 and MG1693+pBR325 optical density measurements at 620 nm for determination of the growth curves, in LB medium at 37º C and 120 rpm. (b) Viability evaluated by number of colony forming units in properly supplemented LA plates for the same strains and conditions.

starter culture (Fig. 5A). In contrast, MG1693 transformed with pMAK580 strongly depends on the conditions cultures were exposed before inoculum was diluted in new media [12]. The overexpression of *ibaG* from pBGA01 showed a deleterious effect as evaluated in the growth curve (Fig. 5A); nevertheless the outcome was significantly less notable than pMAK580 effect under the same conditions (Guinote et al., unpublished results). The viabilities were assessed in the different strains (Fig. 5B). The presence of pBR325 does not change viability results except at mid exponential phase. The number of dividing cells is similar to MG1693 wt strain for all the other time points analyzed. Similarly to what was observed in the growth curves, viability results show that overexpression of *ibaG* by pBGA01 plasmid was in general detrimental, with the sole exception of the transition to stationary phase; this plasmid significantly reduces colony counts. Also in pMAK580 transformed strain, the viabilities were always quite low and constant (Fig. 5B).

Furthermore, in *Escherichia coli*, when the *bolA* morphogene is overexpressed not only cell division is affected but cells become rounder. Therefore we have evaluated the morphological phenotype caused by the presence of the plasmid pBGA01. Conversely to what happens with *bolA* no morphological changes were observed in the presence of *ibaG* overexpression (data not shown). Carbon depletion stress was also tested, however, only pMAK580 transformed strain showed spherical morphologies; the pBGA01 transformed strain behaved similarly to the wild type MG1693. Therefore the increased copy number of *ibaG* does not reduce the viabilities through a mechanism that involves morphological changes and thus cannot be monitored in that way – unlike *bolA*, *ibaG* is not a morphogene.

ibaG is not regulated by BolA but seems to require its presence for regular transcription

According to the previous results *ibaG* and *bolA* seem

Table 2.

	ka (1/Ms)	kd (1/s)	KA (1/nM)	KD (nM)	Chi2	ΔG^0 (KJ/mol)
***ibaG* prom**	285	1,00E-05	0,028	35,20	0,59	-42,54
***ibaG* ORF**	616	1,04E-05	0,059	16,90	0,96	-44,36

Table 2. BolA binding affinity for *ibaG* promoter (*ibaG* prom) and coding region (*ibaG* ORF). Equilibrium constants (K_D) were determined by Surface Plasmon Resonance using BIACORE2000 and according to the 1:1 Langmuir Binding Model. k_a is the association rate constant, k_d the dissociation rate constant, and K_D the equilibrium dissociation constant of the reaction. χ^2 was the statistics used to measure the fitness of the model to the data. ΔG^o values were determined according to the van´t Hoff equation: $\Delta G^{\circ} = RT\ln K_D$, where R and T are the universal gas constant and absolute temperature.

to concur in distinct pathways. Since BolA was shown to interact with the promoter regions of *mreB*, *dacA* and *dacC*, by Surface Plasmon Resonance (SPR), the same methodology was used to test the ability of BolA to recognize and interact with the *ibaG* promoter and open reading frame (ORF) regions. The results indicate similar weak binding affinities of BolA to both nucleic sequences (Table 2). Thus, under these conditions, BolA is established not to act as a direct transcriptional regulator for *ibaG*. Nevertheless, any indirect influence on transcriptional change cannot be detected by such experiment. Therefore *ibaG* transcription dynamics according to BolA levels was evaluated by using a GFP reporter gene fused with *ibaG* promoter region. The pRMA02 plasmid was constructed with *gfp* being expressed according to the *ibaG* upstream promoter activity. In this methodology, transcription activity of the cloned promoter(s) is measured by determination of GFP fluorescence per cell. Cells were transformed with pRMA02 and pPFA02 [9] (*bolA* overexpression plasmid) and GFP fluorescence was measured. Transcription from *ibaG* promoter did not reveal significant variations due to increased BolA levels. However, transcription levels of *ibaG*, measured by fluorescent GFP, were halved when *bolA* was not induced (Fig. 6A). Hence, BolA seems to be required for the correct transcriptional activity of *ibaG* promoter. In order to confirm this hypothesis wt and *bolA* deletion isogenic strains were also transformed with pRMA02 plasmid and the transcription activity of *ibaG* promoter was evaluated in both backgrounds (Fig. 6B). The transcription

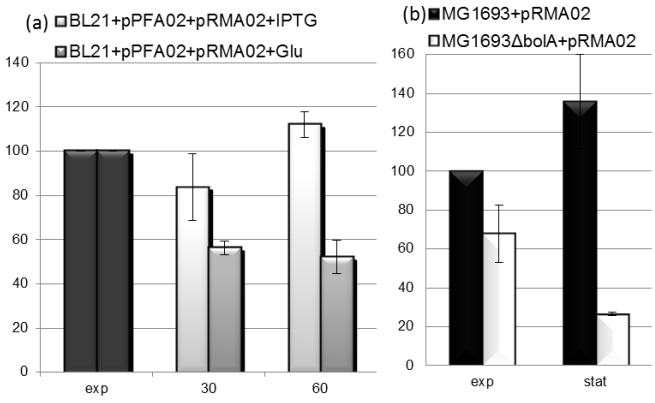

Figure 6. *ibaG* transcription evaluation (a) Measurements on the *bolA* inducible strain BL21+pPFA02+pRMA02, at 0 (OD 0.5), 30 and 60 minutes after addition of either glucose (repressing *bolA* leakage expression) or IPTG (inducing *bolA*). (b) Measurements on MG1693+pRMA02 and isogenic *bolA* deletant strain, at mid exponential and late stationary phase. The averages of GFP fluorescence per cell (by EF-Tu quantification) were determined as a percentage of the exhibited at exponential fase (OD 0.4).

activity of *ibaG* promoter in *bolA* mutant decreased 30% in mid exponential phase and 70% in stationary phase. When *bolA* is physiologically expressed at lower levels, the difference of *ibaG* transcription between the wild type and the *bolA* deletion strain is much lower than in stationary phase, when the expression of *bolA* is physiologically more significant. BolA is therefore shown to be necessary to maintain regular *ibaG* transcriptional levels, with a strong emphasis in stationary phase of growth, when BolA is normally present in increased concentration.

ibaG mRNA expression responds to acid stress

Both *bolA* and *ibaG* patterns of expression along the growth curve and upon stresses imposition were evaluated by Northern blot. The *bolA* mRNA levels were only increased in the strain transformed with pMAK580, the *bolA* overexpression plasmid. The transcript was absent in the deletion strain, as expected and the levels of *bolA* mRNA in the wild

type strain, the single *ibaG* deletant mutant, the strain with the control vector pBR325, and in the strain with the *ibaG* overexpressing pBGA01 plasmid were approximately the same. As a result, we could conclude that increased IbaG levels did not influence the regulation of *bolA* gene (Fig. 7A).

When we used an *ibaG* probe for equivalent membranes, it could not be detected (data not shown). Nevertheless, the expression of the *ibaG* gene was possible to evaluate in the strain transformed with pBGA01 plasmid, where the gene is still controlled by its own promoter but in higher copy number (Fig. 7B). This plasmid was constructed with the *ibaG* gene plus the 305 bp region that follows to prevent regulatory regions of the RNA to be cut. The ibaG gene should be of about 320 bp in size. The presence of an additional band of about 625 bp in the pBGA01 plasmid transformed strain further confirmed the co-transcription of *ibaG* and *murA*. Since it is derived from pBR325, this plasmid is present until five times more in stationary phase. The levels of *ibaG* transcripts were nevertheless the lowest in stationary phase, which means that the gene is almost absent at this growth phase. The highest expression of this gene occurs at mid exponential phase, when *bolA* mRNA levels start increasing.

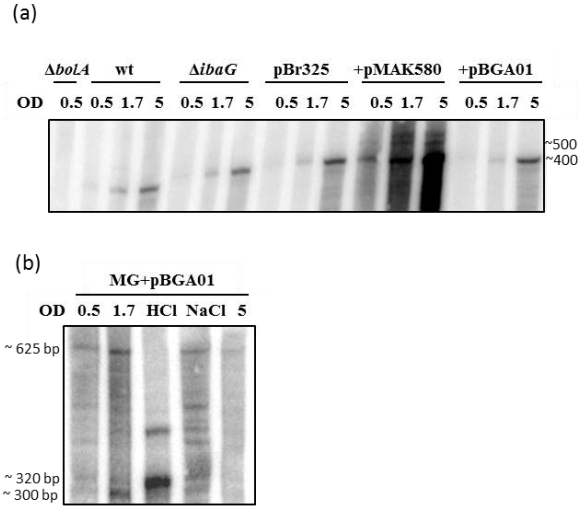

Figure 7. Northern Blot transcriptional analysis of *bolA* and *ibaG* according to the presence and levels of the homologue proteins, growth phases and imposed stresses. (a) *bolA* mRNA in MG1693Δ*bolA* (negative control), MG1693, MG1693Δ*ibaG*, MG1693+pBr325, MG1693+pMAK580, and MG1693+pBGA01, respectively, at OD 0.5, 1 hour after OD 0.5 ~ 1.7, and 5, corresponding to early exponential, mid exponential, and stationary phases. (b) *ibaG* mRNA at early exponential phase (OD 0.5), mid exponential phase (1 hour after OD 0.5 ~ 1.7), one hour of acid stress (HCl), one hour after osmotic stress (NaCl), and stationary phase (OD 5), respectively. This transcript only be detected in the *ibaG* overexpression strain, MG1693+pBGA01.

Two different stresses were checked, the osmotic and acid stress. When osmotic stress is imposed *ibaG* expression is not shut down, but the mRNA seems to be present in significant lower levels compared to the standard growth or acidic conditions. When cells are challenged with hydrochloric acid stress, *ibaG* levels shift and a different mRNA pattern can be observed (Fig. 7b). Since *ibaG* mRNA expression responds so strongly to acid stress, we decided to rename this gene, formerly *yrbA*, as *ibaG* for "**i**nfluenced **b**y **a**cid **g**ene".

ibaG favours growth in acidic conditions

After the transcriptional analysis results, *ibaG* was postulated to be involved in the survival or growth enhancement in acid conditions. The strains growths were monitored upon acid and osmotic challenge. As expected, no differences were acknowledged between genotypes when different osmotic pressures were applied (data not shown). The evaluation for acid challenge also did not differ for pH 3 and 4 – in those

extreme acid conditions all the *E.* coli strains stopped growing (data not shown). All the same, when neutral cultures were switched to LB at pH5, the strain overexpressing *ibaG* grew better than the wild type and, conversely, the deletion strain was more sensitive to acid stress (Fig. 8). Between 180 and 240 minutes after the stress challenge, the strains ODs basically over imposed and gradually diverged from that time point on with inverted pattern to what happened at neutral pH in LB. The strain overexpressing *ibaG* grows significantly better than the mutant or even wild type. Therefore, *ibaG* is shown to be involved in tolerance against mild acid environments (pH5). *IbaG* mRNA is increased in exponential phase, upon acid stress imposition, and was shown to contribute to *E. coli* tolerance against acid stress.

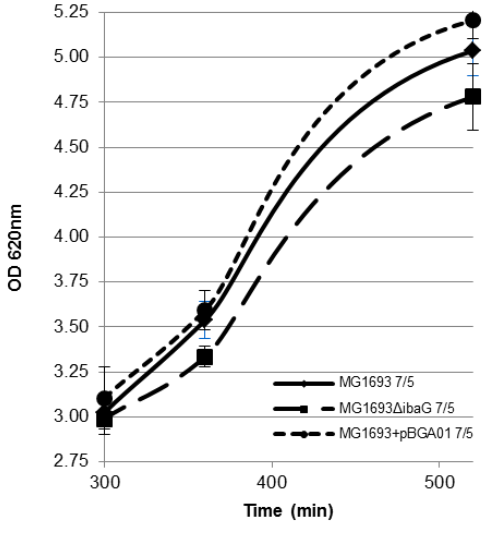

Figure 8. Growth behavior of MG1693, MG1693Δ*ibaG*, and MG1693+pBGA01, determined for pH5 acid stress challenge induced after OD 0.4/0.5 was reached by all cultures. MG1696+pBr325 strain grew similarly to the deletion strain (data not shown).

Final Remarks

In this report, we have shown that IbaG (former YrbA) is a BolA homologue protein in *Escherichia coli*, with significant amino acid sequence similarity. The initial hypothesis that this homologue could replace *bolA* in a single deletion mutant was discarded. Both *bolA* and *ibaG* were confirmed not to be essential genes. The single *ibaG* (as the double) deletion mutant grow better than the wild type and, in turn, the increased *ibaG* copy number strain presents decreased growth and viabilities, in rich neutral medium. Cells growing with this plasmid do not produce the morphology phenotypes related to increased *bolA* levels. *IbaG* levels do not affect *bolA* transcript levels. Reversely BolA seems to be crucial for *ibaG* to be properly transcribed. While most of the *bolA* known phenotypes are not reproduced by *ibaG*, this gene is responsive to acid stress, and was thus named *ibaG*, "**i**nduced **b**y **a**cid **g**ene". Upon pH5 acid challenge, the *ibaG* overexpression strain grew better than the wt and the *ibaG* deletion strain, indicating that this gene is involved in resistance and survival against acid stress.

Acknowledgements

We would like to thank Keio University [4] for all their kindness providing us the deletion strains for *bolA* and *ibaG*. IBG and RNM were recipients of Doctoral fellowships from FCT (Fundação para a Ciência e Tecnologia - Portugal). We would like to acknowledge our technician Andreia Aires for her

technical support in the lab. We thank FCT for funding of this project and Instituto Gulbenkian de Ciência for the access to their Cellular Imaging Unit, where all microscopy studies were performed.

Bibliography

1. Aldea, M., T. Garrido, C. Hernandez-Chico, M. Vicente, and S. R. Kushner. 1989. Induction of a growth-phase-dependent promoter triggers transcription of bolA, an Escherichia coli morphogene. *EMBO J* **8:** 3923-3931.
2. Aldea, M., T. Garrido, J. Pla, and M. Vicente. 1990. Division genes in Escherichia coli are expressed coordinately to cell septum requirements by gearbox promoters. *Embo J* **9:** 3787-3794.
3. Aldea, M., C. Hernandez-Chico, A. G. de la Campa, S. R. Kushner, and M. Vicente. 1988. Identification, cloning, and expression of bolA, an ftsZ-dependent morphogene of Escherichia coli. *J Bacteriol* **170:** 5169-5176.
4. Baba, T., T. Ara, M. Hasegawa, Y. Takai, Y. Okumura, M. Baba, K. A. Datsenko, M. Tomita, B. L. Wanner, *et al.* 2006. Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol* **2:** 2006 0008.
5. Bearson, S., B. Bearson, and J. W. Foster. 1997. Acid stress responses in enterobacteria. *FEMS Microbiol Lett* **147:** 173-180.
6. Brown, E. D., E. I. Vivas, C. T. Walsh, and R. Kolter. 1995. MurA (MurZ), the enzyme that catalyzes the first committed step in peptidoglycan biosynthesis, is essential in Escherichia coli. *J Bacteriol* **177:** 4194-4197.
7. Datsenko, K. A. and B. L. Wanner. 2000. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proc Natl Acad Sci U S A* **97:** 6640-6645.
8. Freire, P., J. D. Amaral, J. M. Santos, and C. M. Arraiano. 2006. Adaptation to carbon starvation: RNase III ensures normal expression levels of bolA1p mRNA and sigma(S). *Biochimie* **88:** 341-346.
9. Freire, P., R. N. Moreira, and C. M. Arraiano. 2009. BolA inhibits cell elongation and regulates MreB expression levels. *J Mol Biol* **385:** 1345-1351.
10. Freire, P., H. L. Vieira, A. R. Furtado, M. A. de Pedro, and C. M. Arraiano. 2006. Effect of the morphogene bolA on the permeability of the Escherichia coli outer membrane. *FEMS Microbiol Lett* **260:** 106-111.
11. Gama-Castro, S., H. Salgado, M. Peralta-Gil, A. Santos-Zavaleta, L. Muniz-Rascado, H. Solano-Lira, V. Jimenez-Jacinto, V. Weiss, J. S. Garcia-Sotelo, *et al.* RegulonDB version 7.0: transcriptional regulation of Escherichia coli K-12 integrated within genetic sensory response units (Gensor Units). *Nucleic Acids Res* **39:** D98-105.
12. Guinote, I. B., R. G. Matos, P. Freire, and C. M. Arraiano. 2011. BolA affects growth and binds to the promoters of Penicillin-Binding Proteins 5 and 6 regulating their expression. *Journal of Microbiology and Biotechnology* **21(3):** 243-251.
13. Herring, C. D. and F. R. Blattner. 2004. Conditional lethal amber mutations in essential Escherichia coli genes. *J Bacteriol* **186:** 2673-2681.
14. Kasai, T., M. Inoue, S. Koshiba, T. Yabuki, M. Aoki, E. Nunokawa, E. Seki, T. Matsuda, N. Matsuda, *et al.* 2004. Solution structure of a BolA-like protein from Mus musculus. *Protein Sci* **13:** 545-548.
15. Kim, M. J., H. S. Kim, J. K. Lee, C. B. Lee, and S. D. Park. 2002. Regulation of septation and cytokinesis during resumption of cell division requires uvi31+, a UV-inducible gene of fission yeast. *Mol Cells* **14:** 425-430.
16. Lange, R. and R. Hengge-Aronis. 1991. Growth phase-regulated expression of bolA and morphology of stationary-phase Escherichia coli cells are controlled by the novel sigma factor sigma S. *J Bacteriol* **173:** 4474-4481.
17. Lin, J., I. S. Lee, J. Frey, J. L. Slonczewski, and J. W. Foster. 1995. Comparative analysis of extreme acid survival in Salmonella typhimurium, Shigella flexneri, and Escherichia coli. *J Bacteriol* **177:** 4097-4104.
18. Malinverni, J. C. and T. J. Silhavy. 2009. An ABC transport system that maintains lipid asymmetry in the gram-negative outer membrane. *Proc Natl Acad Sci U S A* **106:** 8009-8014.
19. Marquardt, J. L., D. A. Siegele, R. Kolter, and C. T. Walsh. 1992. Cloning and sequencing of Escherichia coli murZ and purification of its product, a UDP-N-acetylglucosamine enolpyruvyl transferase. *J Bacteriol* **174:** 5748-5752.
20. Martinez-Antonio, A., S. C. Janga, and D. Thieffry. 2008. Functional organisation of Escherichia coli

transcriptional regulatory network. *J Mol Biol* **381:** 238-247.

21. Miksch, G. and P. Dobrowolski. 1995. Growth phase-dependent induction of stationary-phase promoters of Escherichia coli in different gram-negative bacteria. *J Bacteriol* **177:** 5374-5378.
22. Miller, J. H. 1972. Experiments in Molecular Genetics, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
23. Muffler, A., D. D. Traulsen, R. Lange, and R. Hengge-Aronis. 1996. Posttranscriptional osmotic regulation of the sigma(s) subunit of RNA polymerase in Escherichia coli. *J Bacteriol* **178:** 1607-1613.
24. Reese, M. 2000. Computational prediction of gene structure and regulation in the genome of Drosophila melanogaster''.
25. Sambrook, J., Maniatis, T., and Fritsch, E.F. 1989. Molecular cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor,NY: Cold Spring Harbor Laboratory Press.
26. Santos, J. M., D. Drider, P. E. Marujo, P. Lopez, and C. M. Arraiano. 1997. Determinant role of E. coli RNase III in the decay of both specific and heterologous mRNAs. *FEMS Microbiol Lett* **157:** 31-38.
27. Santos, J. M., P. Freire, F. S. Mesquita, F. Mika, R. Hengge, and C. M. Arraiano. 2006. Poly(A)-polymerase I links transcription with mRNA degradation via sigmaS proteolysis. *Mol Microbiol* **60:** 177-188.
28. Santos, J. M., P. Freire, M. Vicente, and C. M. Arraiano. 1999. The stationary-phase morphogene bolA from Escherichia coli is induced by stress during early stages of growth. *Mol Microbiol* **32:** 789-798.
29. Santos, J. M., M. Lobo, A. P. Matos, M. A. De Pedro, and C. M. Arraiano. 2002. The gene bolA regulates dacA (PBP5), dacC (PBP6) and ampC (AmpC), promoting normal morphology in Escherichia coli. *Mol Microbiol* **45:** 1729-1740.
30. Vieira, H. L., P. Freire, and C. M. Arraiano. 2004. Effect of Escherichia coli morphogene bolA on biofilms. *Appl Environ Microbiol* **70:** 5682-5684.