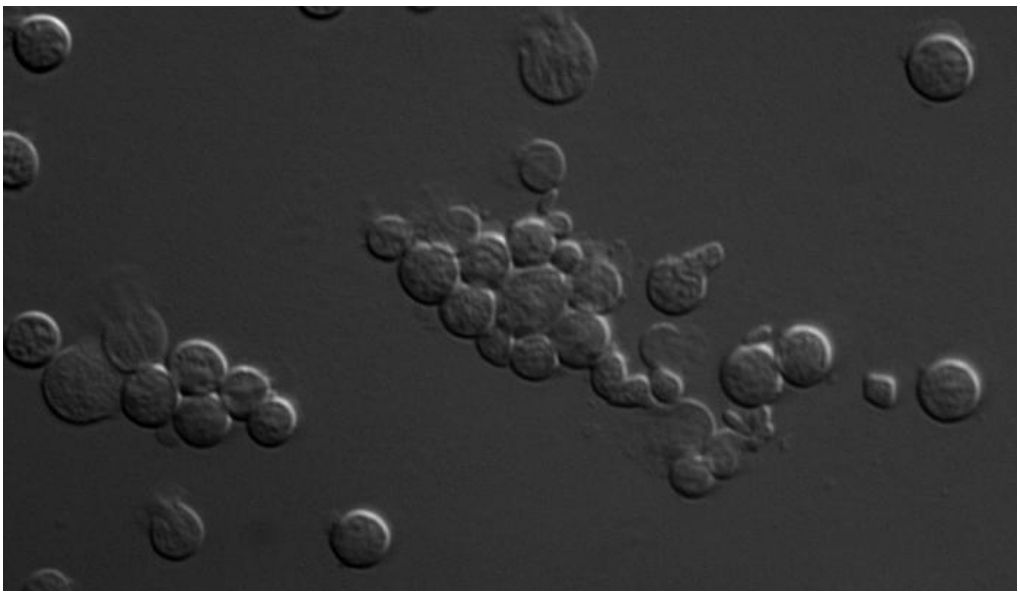
Instituto de Tecnologia Química e Biológica

Universidade Nova de Lisboa

Functional studies on BolA and related genes:

increasing the understanding of a protein with pleiotropic effects



Inês Batista Guinote

Oeiras, 28th January 2011

“DEVAGAR, MAS SEMPRE

“Mas ainda que o nosso homem exterior se corrompa,

o interior, contudo, se renova, de dia para dia.”

***Paulo* (II CORÍNTIOS, 4:16)**

Observa o espírito de sequência e gradação que prevalece nos mínimos setores da Natureza. Nada se realiza aos saltos e, na pauta da Lei Divina, não existe privilégio em parte alguma.

Enche-se a espiga de grão em grão. Desenvolve-se a árvore milímetro a milímetro. Nasce a floresta de sementes insignificantes. Levanta-se a construção peça por peça. Começa o tecido nos fios. As mais famosas páginas foram produzidas letra a letra. A cidade mais rica é edificada palmo a palmo. As maiores fortunas de ouro e pedras foram extraídas do solo, fragmento a fragmento. A estrada mais longa é pavimentada metro a metro. O grande rio que se despeja no mar é conjunto de filetes líquidos.

Não abandones o teu grande sonho de conhecer e fazer, nos domínios superiores da inteligência e do sentimento, mas não te esqueças do trabalho pequenino, dia a dia (…) é indispensável saibamos perseverar com o esforço de auto-aperfeiçoamento, em vigilância constante, na atividade que nos ajude e enobreça. (…)

Há ensejo favorável à realização? Age com regularidade de alma voltada para a meta. Há percalços e lutas, espinhos e pedrouços na senda? Prossegue mesmo assim.

O tempo, implacável dominador de civilizações e homens, marcha apenas com sessenta minutos por hora, mas nunca se detém.

Guardemos a lição e caminhemos para diante, com a melhoria de nós mesmos.

DEVAGAR, MAS SEMPRE!”

in **Fonte Viva**, Francisco Cândido Xavier, ditado pelo Espírito Emmanuel, FEB

Functional Studies on BolA and related genes: increasing the understanding of a protein with pleiotropic effects.

Inês Batista Guinote

Dissertation presented to obtain a Doctoral degree in Biology by Instituto de Tecnologia Química e Biológica

Instituto de Tecnologia Química e Biológica
Universidade Nova de Lisboa
Oeiras, January 2011

ISBN: 978-989-20-2347-2

Inês Batista e Guinote was the recipient of a Doctoral Fellowship from **Fundação para a Ciência e Tecnologia (FCT):** PhD grant – SFRH/BD/ 31758/2006. The work was suspended for 5 months for maternity leave.

Work performed at:

Control of Gene Expression Laboratory

Instituto de Tecnologia Química e Biológica

Av. da República (Estação Agronómica Nacional)

2781-901 Oeiras – Portugal

Telf: +351 214469562

Fax: +351 214469549

http://www.itqb.unl.pt/Research/Biology/Control_of_Gene_Expression

Supervisor:

Professora Doutora Cecília Maria Pais de Faria de Andrade Arraiano – Investigadora Coordenadora, Instituto de Tecnologia Química e Biológica, Universidade de Lisboa.

(Head of the Control of Gene Expression Laboratory, where the work of this Dissertation was performed)

Co-Supervisor:

Doutor Patrick de Oliveira Freire – Investigador do Laboratório Nacional de Investigação Veterinária, Instituto Nacional de Recursos Biológicos.

President of the Jury:

Professora Doutora Maria Helena Dias dos Santos – Investigadora Principal, Instituto de Tecnologia Química e Biológica, Universidade de Lisboa.

(Head of the Cell Physiology and NMR Laboratory,)

Examiners:

Professor Doutor Arsénio do Carmo Sales Mendes Fialho – Professor Associado do Instituto Superior Técnico, Universidade Técnica de Lisboa *(Principal Examiner).*

Doutor Jorge Miguel Pereira de Oliveira da Silva Santos – Investigador EcBio Cell Biotechnologies Chief Operations Officer (COO) *(Principal Examiner).*

.

Professor Doutor Jorge Humberto Gomes Leitão –Professor Auxiliar do Instituto Superior Técnico, Universidade Técnica de Lisboa.

Group photo with examiners. Professor Doutor Arsénio Fialho, Professor Doutor JorgeLeitão, Doutor Patrick Freire, Inês Batista Guinote, Professora Doutora Cecília Maria, Doutor Jorge Miguel Santos, Professora Doutora Helena Santos.

Control of Gene Expresssion lab group. Joana Pissara, Vânia Pobre, Sandra Viegas, Clèmentine Dressaire, José Andrade, Michal Malecki, Inês Batista Guinote, Susana Domingues, Andreia Aires, Inês Silva, Cátia Bárria, Ana Filipa Reis, Doutora Cecília Arraiano, Ricardo Neves Moreira, Rute Gonçalves Matos, Margarida Saramago.

To all of my Family:

mamã, papá, mano, madrinha, padrinho, afilhada…

Hugo, Gonçalo, Clara, G.

(in loving memory of avó Rosária)

“Happiness demands bravery (…)

To be happy… is to stop being a victim of external problems

and to become the author of your own story (…)

Stones in the way?

I save them all… One day I will build a castle!”

adapted from Augusto Cury

Acknowledgments

I would like to thank all the conditions given to me and even more importantly, all the people that directly or indirectly contributed and supported my PhD thesis, in the absence of which it would not exist:

First of all, I would like to acknowledge **Instituto de Tecnologia Química e Biológica**, for its excellence, for the fantastic opportunity of working where most instruments are available and updated, something that is vital for a good workflow, and where so many groups interact and help each other in knowledge transfer, equipment lending or even specific reagents supply; where facilities have both space and light, and even geographic proximity to the sea, visited by our group for several times throughout this dissertation work, usually celebrating so many personal as well as professional achievements… I would like to thank particularly to the former **Director Professor Doutor Miguel Teixeira** for accepting me in the institution and providing the conditions to work and to the present **Director Professor Doutor José Artur Martinho Simões** for the effort to improve the facilities, equipment and management, namely accepting **Doutora Cláudia Almeida** as a new and great asset revealed Lab Manager, and for allowing the presentation and discussion of the present dissertation. Finally, I would like to recognize **Doutora Susana Neves**, our dearest librarian for her help in getting so many and vital literature for protocols and overall data.

Also vital for the work here performed was the contribution of **Fundação para a Ciência e Tecnologia (FCT)** in the individual grant provided in my name SFRH / BD / 31758 / 2006, but also crucial for the implement and work development through the BolA related projects wan by the lab where the work was performed.

Leading my introduction in this institute, writing the grant project for my PhD application, giving all the means ever request for work to proceed, providing the chance to rotate between so many different lab tasks and learn them all, always attentive for tiredness or family responsibilities I had, I hereby present homage to **Professora Doutora Cecília Maria Arraiano**, the leader of **Control of Gene Expression Laboratory**. More than an extraordinary awards winning motivational lab leader, Doutora Cecília was a PhD supervisor truly decisive for my life plans, pushing hard on me to speedily come up with a dissertation to present in due time, avoiding to endanger any family commitments. My most *strongly*, *deeply*, *truly* thanks.

To all of my lab colleagues: **Ana Barbas** and **Patrick Freire**, allocated with a merited position, the post-docs **José Andrade**, **Sandra Viegas**, **Susana Domingues**, **Michal Malecki**, and **Clèmentine Dressaire**, the PhD students **Rute Margarida Matos**, **Ricardo Neves Moreira**, **Inês Silva**, **Ana Filipa Reis, Ana Margarida Saramago**, **Cátia Bárria**, and **Joana Pissarra**, the former master colleague **Ana Margarida Matos**, that is now happily working in the industry, and our dearest and most effusive technician **Andreia Aires**… a special thank you!!! For the patience when I would drink coffee and completely accelerate, for caring when my baby girl was born and when me or my owns were sick, for being there, even if only just to give support, when things would go bad in work - when enzymes or tests or methodologies would not cooperate; when tests would finally work and results were nevertheless negative, again leading to dead ends!!- More recently, for your interest in the completion of the work and writing; for your belief in the awareness of the results; for proposing to help in which ever I would need, spending our most precious treasure – time; and for your leap of faith with me (and not apart from me) into a better end than path.

x

More particularly involved in my life and work here developed: a thank you kiss to **Patrick** for guiding the work here described, for teaching, for providing the protocols to use but, even more, for making me search for them and become independent, for analyzing results and sometimes put things in perspective reducing the load; the warmest hug to **Rute**, *luckily*, my companion ever since university throughout all the degree, and until today, again we meet "by chance" if we would believe in such a thing… for being my pal, my confident, my hands and my head (when mine was so tired that my body was more driven by the feet then it), for giving me the best advice ever, for urging me to go to the bench if I was sinking in the obscurities of the work, or to stop creating new experiments to run and instead write, to submit papers in due time; for hearing ALL of my problems and difficulties without complaint or revealing annoyed, for always being there… I'll be eternally yours… you will always be in my heart; **Ricas**, fingers crossed for this *ibaG* paper… I will never forget you, and I hope that your thesis does not have to involve the amount of running and stress mine did… best wishes; light breeze, fresh air, big teen, the joy of this lab nowadays, our darling **Andreia**, came in the end to "partially substitute" me from being the "lab technician" – yet I really enjoyed the possibility of helping others, and to facilitate anyone's work… give a little bit of myself to all, even if only in really small lab stuff – you have additionally involved yourself in my latest work with the sole purpose of helping me in my time of need… I hope these results become the best and most promising I've been involved so far, for you to add it to you CV and gain your *Licenciatura* in pharmacy (?), one paper ahead of all others! **Cátia**, a saddening event made our encounter too brief… I truly hope that our path joins again in the future! Although we have only been together the last few months I fell as if I have known you forever… the shared laughter and cheerful moments will never be forgotten! To the lab, lots and lots of kisses!

To **all** the other **ITQB lab's colleagues**, especially those on the 5th floor and of the glicobiology lab with whom we have shared difficult as well as extremely happy moments, and that were always available to give a hand, particularly **Ana Jorge**, **Magda Atilano**, **James Yates**, **Tânia Ribeiro**, **Marta Abrantes**, **Luciana Pereira**, **Isabel Correia**, and **Ricardo Gouveia**… To the washing room ladies **Pilar Campos**, **Carmen Fernandes**, **Helena Vilaranda**, **Sónia Moita**, **Alice Ferreira** and their responsible **Teresa Batista da Silva,** for their fine work and availability when an excess of demands were asked (and those happened quite sometimes). To the security guards, for their company when working late nights or weekends, and joking words to maintain the joy… To all the accounting department, the academic office, maintenance and storage services, particularly **João Rodrigues**, **Carlos Martins** and **Bruno Gouveia** with whom contact was constant! All the best!

To **Instituto Gulbenkian Ciência (IGC)**, particularly the cell imaging unit, in the persons of **Nuno Moreno**, **Bárbara Beltrame**, and **Gabriel Martins** for the technical and instrumental support and opening doors for courses and work.

To all of my **friends** outside ITQB and science, thank you for worrying, for caring, for knowing and want to know! My heart is yours… forever… whenever…

To my family… **Gabrieis**, **Batistas** (**Alves**), **Guinotes** for your eternal support and unconditional love…

To **Luisa** and **Tó** for your help with the kids and at home…

To **Mum**, **Dad**, and **Baby Brother**, for your presence in my life… past, present, and future… you are everything!...

To **Hugo**, for your patience… for our life… for our love… for our offspring…

To **Gonçalo**, **Clara**, **G.** for existing… for the shared laughter and fun…

ALL of my LOVE…

Table of contents

List of publications

Scientific articles in international peer reviewed Journals:

GUINOTE I. B., Matos R. G., Freire P., and Arraiano C. M., **BolA affects growth and binds to the promoters of Penicillin-Binding Proteins 5 and 6 regulating their expression**, Journal of Microbiology and Biotechnology, *in press*.

Arraiano C. M., Andrade J. M., Domingues S., GUINOTE I. B., Malecki M., Matos R. G., Moreira R. N., Pobre V., Reis F. P., Saramago M., Silva I. J., Viegas S. C., **The critical role of RNA processing and degradation in the control of gene expression**, FEMS Microbiology Reviews (2010), 34(5):883-923.

GUINOTE I. B., Moreira R. N., Freire P., and Arraiano C. M., **Characterization of the BolA homologue *ibaG*: a new gene involved in acid resistance**, *to be submitted*.

GUINOTE I. B., Moreira, R. M., Freire P., and Arraiano C. M., **Gram-negative cell wall regulation and BolA mediated protection against stresses in *E. coli***, *to be submitted*.

GUINOTE I. B., Freire P., and Arraiano C. M., **BolA can contribute to dormancy, protecting cells against external stresses *in E. coli*: is BolA a new persister gene?** *in preparation*.

Dissertation outline

This dissertation is divided into seven chapters.

In the first chapter is an introduction about several of the aspects that may concern the study of BolA. This introduction incorporates all of the aspects already known about BolA.

The second chapter shows that overproduction of BolA affects bacterial growth differently depending on whether the cells are inoculated directly from plate or from overnight batch cultures. It furthermore demonstrates that BolA is a transcriptional regulator of the *dacA* and *dacC* genes, thus regulating both DD-carboxypeptidases PBP5 and PBP6. It is shown that some BolA cellular effects depend on PBP5 or PBP6.

In the third chapter we have evaluated BolA as a putative persistence protein, inducing a decline in the multiplication potential but increasing toleration against different stresses imposed.

In the fourth chapter the BolA homologue protein YrbA (renamed as IbaG) was studied as a possible BolA protein homologue. Although these two proteins did not induce similar phenotypes, they both induced a decrease in growth and viability when overexpressed, and increased ODs and cell counts in the respective deletion strains. IbaG did not change the morphologies of cells in any of the conditions tested. This gene is in an operon with the essential *murA* gene. IbaG is induced in acid stress conditions, and therefore was renamed as **i**nduced **b**y **a**cid **g**ene. It has furthermore conferred advantages for growth upon mild acid challenge.

In the fifth chapter a General Discussion puts in perspective the new information presented throughout the above chapters.
In the sixth chapter perspectives are provided regarding the future work on BolA, taking into account several other mechanisms that BolA might interfere with. General perspectives give suggestions that can be developed by other lab colleagues currently working on BolA.

The seventh chapter is an appendix presenting some preliminary work related to BolA, that has been started but is still incomplete. In Appendix I the role of Hfq and glucose are evaluated in *bolA* regulation. In Appendix II we have developed tools to evaluate *E. coli bolA* in pathogenic species belonging to the *Burkholderia cenocepacia* complex.

A lot more work needs to be implemented into true knowledge about BolA; it is my conviction that this molecule has still a lot to offer. But, like a precious untouched diamond, it needs a gigantic amount of ability, work and most of all patience to set it free.

Good journey!

Abbreviations

Amp ampicillin

Bcc Burkholderia cepacia complex

bp base pair

bolA1p promoter P1 of *bolA* gene

bolA2p promoter P2 of *bolA* gene

BSA bovine serum albumin

ºC degree Celsius

Cam chloramphenicol

cfu colony forming units

CF cystic fibrosis

Δ deletion

Da dalton

DTT dithiothreitol

dATP 2′-deoxyadenosine 5′-triphosphate

DNA deoxyribonucleic acid

DNase deoxyribonuclease

E.coli *Escherichia coli*

EDTA EthyleneDiamineTetraAcetic acid

EPS exopolysaccharide

h hour

HMM High Molecular Mass

IPTG IsoPropyl-β-D-thiogalactopyranoside

Kan kanamycin

kb kilobase

K_D dissociation constant

kDa kilodalton

L liter

LB Luria-Bertani Broth

LMM Low Molecular Mass

log logarithm

M molar/molarity(mol/L)

M9 M9 minimal medium

mg milligram

µg microgram

µl microliter

µM micromolar

min minute

ml milliliter

mM millimolar

mmol millimole

mol mole

mRNA messenger RNA

MW molecular weight

Nal nalidixic acid

ng nanogram

nM nanomolar

nm nanometer

nmol nanomole

nt nucleotide

OD optical density

o.n. overnight

OM outer membrane

ORF open reading frame

^{32}P phosphorus 32 radionucleotide
PAA polyacrylamide
PAP I Polyadenilation Polymerase I
PAGE polyacrylamide gel electrophoresis
PBP penicillin-binding-protein
PBS phosphate buffered saline buffer
PCR polymerase chain reaction
PG peptidoglycan
pmol picomole
PNPase PolyNucleotide Phosphorilase
psi pressure unit
RNA ribonucleic acid
RNase ribonuclease
RNaseII ribonuclease II
RNaseIII ribonuclease III
rpm rotations per minute
rRNA ribosomal RNA
RT reverse transcriptase
s second
SDS sodium dodecyl sulphate
SOB Super Optimal Broth
SOC SOB with glucose
SOS stress response pathway triggered by DNA injuries
sRNA small RNA
SSC sodium chloride and sodium citrate buffer/washing solution
σ^{70} sigma 70 subunit of RNApol (encoded by the *rpoD* gene)
σ^{S} sigma S subunit of RNApol (encoded by the *rpoS* gene)
Tet tetracycline
Thy thymine
Tmp trimethropim
Tris trishydroxymethyl aminomethane [2-amino-2-(hydroxymethyl) propane-1,3-diol]
tRNA transfer RNA
UTP uracil triphosphate
UV ultraviolet radiation
V volt
v/v volume/volume
wt wild-type
w/v weight/volume
Z-ring ring of polymerized FtsZ, that forms at the midcell in the beginning of septation

Abstract

BolA is a protein that is able to change bacterial shape, confer resistance against large antibiotic molecules and detergents, reduce permeability, change the equilibrium of the outer membrane porins, and it is even involved in biofilm formation. This protein has such pleiotropic effects, that its function has been very difficult to unravel. This was the starting point for the work of this dissertation. If *bolA* is responsible for global cellular changes that confer resistance to a multitude of stresses, it is imperative to obtain more molecular insights to increase the understanding of the role of BolA in cell physiology and survival.

The first aim of this Doctoral work was to further investigate the relation of BolA with cell wall protein intervenients and cytosqueletal elements. The fact that the levels of BolA in cells can change considerably and the inexistence of proper antibodies for its quantification was an obstacle. Another difficulty was that PBP5 and PBP6 share characteristics: they are quite similar in aminoacidic composition, molecular weight and even isoelectric point. Therefore, there are substancial methodological constraints to separate and evaluate their levels. However we could establish that at least one of them is required for the effectiveness of BolA in cells. In fact, the double deletion mutant for PBP5 and PBP6 was the only strain in which the overexpression of BolA did not lead to the occurrence of spheres. Additionally it was possible to ascertain, by Surface Plasmon Resonance analysis, that BolA is a transcriptional regulator that negatively affects *mreB* and positively affects *dacA* and *dacC*. Although interaction occurs preferentially in the promoter region of these genes (where transcriptional regulation actually takes place) it was reported to occur as well (to a lesser extent) in the Open Reading Frame regions of these genes.

A second objective of this dissertation work was to understand the effects of the expression of BolA on cells growth, morphologies and viabilities. Since BolA is

increasingly expressed along the growth curve, starting the growth at different time points/ODs was expected to correspond to starting growth with different intracellular BolA levels. A work plan was established where starting from OD_{620nm}=0 (directly from colonies), a series of ODs were defined after the first liquid cultures to initiate growth in fresh media. BolA is important enough to be maintained in genomes throughout the living world, nevertheless, when it is present in high levels, it prevents cultures from growing and dividing, strongly reducing their viabilities. This work led to some unexpected results. Even when overexpressed, the BolA-induced slowing of division rate does not seem to represent a killing program or senescence process, given that the strong viability reduction is not progressive along time. Even though BolA seems to prevent division, when *bolA* overexpressing cultures are started with the inoculi at lag or early exponential phase they grow even better than wild type ones. In this way it seems that BolA creates an enhanced growth potential, released upon its clearance from the system. That could be an important mechanism to deal with stress imposition, reducing short term growth but favoring long term resistance and maintenance. This lead us to evaluate BolA potential as a persistence protein, in the way those proteins have been recently studied.

A third objective came after genome wide sequence/structure prediction analysis detected a protein homologous to BolA in *E. coli*. The possibility of a functional substitution by a similar protein projected that a double *bolA*/*yrbA* deletion would not be viable but this work has refuted this idea. In fact, not only the single *yrbA* deletion presents a better growth and viabilities than the wild type, but the double deletion mutant could be constructed and grows even better than the single deletant. Conversely, an increase in the amounts of yrbA (as tested through the construction of pBGA01, pBr325 derived plasmid overexpressing *yrbA*) reduced both growth curves and cell counts. The overexpression of *yrbA* did not

have effects on cells morphologies, again differently of BolA. The mRNA expression pattern was quite low without overexpression but in the presence of the pBGA01 plasmid it could be seen that this transcript is much more present in mid exponential phase than in lag or stationary phase. The response of y*rbA* to stress was studied by Northern Analysis. It was shown that *yrbA* was induced in acid stress conditions. Furthermore the overexpression of *yrbA* led to increased growth upon acid challenge, therefore we named this gene *iba*G, for induced by acid gene. In comparison with the wild type, the *ibaG* deletant strain grew the best in neutral medium but the worst in acid. The *ibaG* overexpressing strain grew worst in pH7 but was the strain which grew the best in pH5. The possibility of *ibaG* levels being controlled by BolA was tested by Surface Plasmon Resonance analysis of interaction, and by transcriptional analysis using GFP protein as signal. Both these methods ruled out of direct interference although it seemed that a minimal *bolA* expression was necessary for proper *yrbA* transcription.

This work proved to be enlightening in the evaluation of BolA as a transcriptional factor, and involved in persistence mechanisms: reduction of metabolism, division, stress protection and boost in growth potential after system clearance. Furthermore, the discovery of IbaG, a BolA related protein, that facilitates survival under acid stress, is quite significant and it opens new perspectives for the understanding of the "BolA family" of genes.

Resumo

A proteína BolA *Escherichia coli* induz a alteração da forma de *bacillus* a *coccus*, confere resistência a detergentes e antibióticos de elevado peso molecular, reduz a permeabilidade, altera o equilíbrio de porinas da membrana externa e ainda induz o desenvolvimento de biofilmes. Apresentando efeitos tão abrangentes e induzindo resistência a uma multitude de stresses, o estudo da função do gene/proteína BolA constituiu o objectivo geral do trabalho que conduziu a esta Dissertação. É imperativo obter conhecimentos de nível fundamental sobre a biologia molecular de BolA, seus alvos, formas de regulação e consequências globais na fisiologia e sobrevivência celulares.

O primeiro objectivo deste trabalho de Doutoramento foi investigar uma eventual relação de BolA com proteínas de membrana e elementos do citosqueleto. A inexistência de bons anticorpos anti-BolA constituiu um obstáculo. Outra dificuldade deveu-se à similitude das proteínas PBP5 e PBP6 - em sequência de aminoácidos, peso molecular e até de ponto isoeléctrico – impossibilitando a determinação dos seus níveis isoladamente. No entanto, foi possivel verificar que os efeitos da presença excessiva de BolA só ocorrem na presença de pelo menos uma das duas DD-carboxipeptidases. De facto, apenas no delectante duplo PBP5/PBP6 com sobreexpressão de *bolA* se conseguiram obter curvas e morfologias semelhantes às das estirpes não transformadas com pMAK580. Além disto, foi possível verificar por *Surface Plasmon Resonance* que o BolA é um regulador transcricional, , actuando como activador para com os genes *dacA* e *dacC*, e como repressor para com *mreB*. Muito embora as interacções tenham sido preferenciais com a região promotora dos genes que regula, verificou-se também interação (menor) com as grelhas abertas de leitura respectivas.

O segundo objectivo desta tese, propôs-se a avaliar as consequências fisiológicas de diferentes níveis de BolA no pré-inóculo de culturas de *E. coli*, procurando

aprofundar os resultados do primeiro estudo, obtidos para crescimentos de estirpes com sobreexpressão de *bolA*. Tendo em conta que ainda não é possível quantificar os níveis proteicos de BolA, e sabendo-se que a expressão deste é aumentada progressivamente com a curva de crescimento, desenvolveu-se um plano de trabalho em que a estirpe com sobreexpressão de *bolA*, CMA10, seria avaliada quanto ao crescimento, viabilidades e morfologias, quando iniciada a partir de diferentes densidades ópticas (ODs). O crescimento líquido desta estirpe seria iniciado a partir de placa (OD_{620nm}=0) e à medida que a cultura atingisse as diferentes ODs predefinidas (0.5, 1.5, 3 e 6) seria utilizada sucessivamente como pré-inóculo para lançamento de novas culturas. Verificou-se que quando em níveis elevados o gene *bolA* dificulta o crescimento das culturas e reduz o número de células viáveis. Ainda assim, este decréscimo de viabilidades não corresponde a um processo de senescência ou morte, tendo em conta que ao longo do tempo as unidades formadoras de colónias não diminuem. Outro resultado surpreendente foi o facto de as mesmas culturas iniciadas após um pré-inóculo em fase lag ou início de exponencial crescerem ainda melhor que as selvagens. A presença de BolA aparenta originar uma repressão temporária de crescimento celular, mas simultaneamente um potencial de crescimento rápido despoletado com o seu desaparecimento do sistema. Esta redução de crescimento a curto prazo e favorecimento a sobrevivência a longo prazo levou-nos também a avaliar o potencial do BolA enquanto hipotética proteína de persistência.

O terceiro objectivo surgiu por análise informática na qual se detectou em *E. coli* uma hipotética proteína homóloga a BolA, designada por YrbA. A possibilidade de substituição funcional do BolA pelo YrbA postulava que o delectante duplo *bolA*/*yrbA* não seria viável, o que foi refutado no âmbito deste trabalho. O delectante simples *yrbA* tem melhor crescimento e viabilidades associadas que a estirpe selvagem. Foi também possível construir o delectante duplo *bolA*/*yrbA*

que ainda apresenta melhores resultados que o delectante simples. Em conformidade com estes resultados, a expressão aumentada de *yrbA* (testada através de construção de pBGA01, um plasmídeo derivado de pBr325, sobreexpressando o *yrbA*) originou um decréscimo tanto nas curvas de crescimento, como nas viabilidades. A expressão de *yrbA* aumentada não originou alterações de morfologia às células em qualquer das condições testadas. A expressão endógena de mRNA mostrou-se demasiado fraca para ser detectada, mas na presença de pBGA01 foi possivel verificar que o transcrito é muito mais expresso a meio da fase exponencial que em fase lag ou estacionária. A resposta do *yrbA* ao stress também foi avaliada por Northern Blot e verificou-se que este é induzido em pH ácido, pelo que foi designado de *ibaG*, traduzindo, gene induzido por ácido. Confirmou-se por SPR e fluorescência (análise transcricional usando a *Green Fluorescent Protein* como sinal) que o BolA não interage com a região promotora do gene em estudo, muito embora a sua presença interfira positivamente para a transcrição do *yrbA*.

O trabalho aqui descrito demonstrou o cumprimento dos objectivos propostos. Comprovou-se a função da proteína BolA como regulador transcricional e a sua importância nos mecanismos de persistência, nomeadamente a redução do metabolismo e da velocidade de multiplicação celular, promoção da tolerância e protecção contra stresses variados e criação de um potencial de crescimento aumentado após o seu desaparecimento do sistema (o que ocorre simultaneamente com o desaparecimento das condições adversas). A caracterização da proteína IbaG homóloga do BolA, a qual se mostrou favorecer a sobrevivência em condições de stress acídico, é muito importante e abre novas portas para a compreensão da "família de genes *bolA*".

Chapter 1

GENERAL INTRODUCTION

INDEX

BolA, a central *E. coli* regulator: Impact on Gram-negative cell wall regulation and adaptation to stresses

Inês Batista Guinote[1], Ricardo N. 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

✉ Correspondence to: Cecília M. Arraiano

Phone: +351 214469547

Fax: +351 214469549

E-mail: cecilia@itqb.unl.pt

Running title: **BolA mediated protection to stresses**

Keywords: BolA, transcriptional regulator, phenotypical differentiation, stress survival, MreB, LMW PBP, gram-negative, cell division

Abbreviations: LMW PBP Low molecular weight Penicillin-binding proteins

ABSTRACT

Microorganisms have continuously evolved to divide and persist in harsh conditions. Bacteria deal with external stresses on a regular basis, many times through major restructuration such as biofilms formation. The adaptation mechanisms usually imply changes in cell shape, roughness, dimensions, protein content, as well as alterations of cell wall structure, thickness and permeability. The *E. coli* morphogene *bolA* is mainly expressed in stress conditions and when overexpressed induces rod bacteria to become spherical. Moreover, BolA is able to induce biofilm formation and to make changes in the outer membrane, which gets less permeable to detrimental agents. Although there is some understanding about the activity of BolA there is no real comprehension of its function on global cell physiology. Does it actually protect from stress? Is that achieved by regulation of other genes? Does it happen in complement with BolA homologues? BolA family of proteins are found throughout the living world with the remarkable exception of the Gram-positive bacteria. This may be due to structural differences in their cell walls (absence of outer membrane, thicker murein layer, presence of teichoic acids), or be somehow related to their exclusive ability to resist stresses through differentiation of spores. Several new questions arise as investigation proceeds into more profound understanding of this interesting family of proteins.

BRIEF BOLA OVERVIEW

Bacteria are able to activate several adaptive responses when facing any form of stress. Alterations of cellular morphology and membrane characteristics are important to regulate the internal exchanges with the environment. Plastic membrane configuration, structure and regulation are beneficial to facilitate solutes and proteins interchange, thus maximizing environmental interactions in optimal growth conditions, and on the other hand promote a differentiation into a more rigid structure, with less porins to ensure permeability reduction, under unfavourable conditions. The gene *bolA* was first described as a stationary phase gene (Aldea et al., 1990; Aldea et al., 1989). Accordingly, the expression of *bolA* is growth phase-regulated and controlled by the sigma factor σ^S (*rpoS* gene) (Lange and Hengge-Aronis, 1991). However, *bolA* has also been established as a general stress response gene induced during early logarithmic growth in response to several stresses, in a partially σ^S-independent manner (Santos et al., 1999). Greater amounts of *bolA* mRNA and a rounder cell morphology were found after application of different sources of stress, such as carbon starvation, osmotic stress, heat shock, acidic stress and oxidative stress (Santos et al., 1999). The *bolA* gene is therefore an *E. coli* morphogene whose product induces spherical shape of the cells when overexpressed, leading to a reduction of the exposed surface (Aldea et al., 1988). This effect of *bolA* on cell morphology is mediated both by the induction of PBP5 and PBP6 D,D-carboxypeptidases (Aldea et al., 1988; Guinote et al., 2010; Santos et al., 2002) and the repression of the expression of MreB (Freire et al., 2009), a bacterial homologue of actin responsible of the formation of an helical cytosqueleton structure required for rod shape configuration in *E. coli* (Carballido-Lopez, 2006). *Escherichia coli* 13.5 kDa BolA protein contains one defined BolA/YrbA domain, essentially formed by a helix-turn-helix (HTH) motif and has recently been confirmed to be a DNA-binding regulator (Aldea et al.,

1989; Freire et al., 2009; Guinote et al., 2010) (Fig.1). It was proven that BolA efficiently binds to the *mreB, dacA* and *dacC* promoter regions. It downregulates *mreB* and upregulates *dacA* and *dacC* promoters, expanding its potential as a transcriptional regulator, with repressor and activator abilities. Moreover, the increased expression of the *bolA* gene 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.

GRAM-NEGATIVE SURVIVAL UNDER STRESS CONDITIONS

Cellular and physiological responses to stress

E. coli cells possess several other mechanisms to cope with nutritional or toxic stresses, besides the abilities to grow in aerobic conditions, perform anaerobic fermentation and even respirate in the absence of oxygen. While many Gram-positive bacteria differentiate long-lasting highly resistant spores, Gram-negative bacteria survive for prolonged periods of starvation or multiple stresses through physiological state adaptations designated as stationary phase. Challenged cells show a variety of adaptations in cellular structure, morphology and physiology, like *flagella*, *pilli* or *fimbriae* development, variations in diameter of the pores of its outer membrane to accommodate larger molecules (nutrients) or to exclude inhibitory substances, synthesizing enzymes to metabolize available compounds or either expel or degrade toxic ones (Halsey et al., 2004). The enhanced stress resistance exhibited by starved bacteria represents a central feature of virulence, since nutrient depletion is regularly encountered by pathogens in their natural *in vivo* and *ex vivo* environments (Frenkiel-Krispin et al., 2001).

Upon sensing an impending saturation level of their population density, *Escherichia coli* cells enter into stationary phase, structurally and functionally

modulating the nucleoid, the transcription apparatus, the translation and proteolytic machinery. Their cytoplasm is condensed and storage compounds and protective substances are synthesized such as glycogen, polyphosphate or trehalose. The volume of the periplasm increases, changes in peptidoglycan composition and structure occur, that result in a higher resistance to autolysis induced by penicillin or chaotropic agents. Cells tend to become smaller, develop an ellipsoid to spherical rather than a rod morphology, and have an increased tendency to form aggregates (Hengge-Aronis, 1999; Hengge-Aronis, 2002). The major DNA-binding proteins, Fis, HU and Hfq, in the exponential-phase nucleoid are replaced by a single stationary-phase protein Dps, and cellular content of the histone-like protein H-NS increases, thereby compacting the nucleoid and ultimately leading to silencing of the DNA functions (Ali Azam et al., 1999) (Ishihama, 1999). The transcription apparatus is modified by replacing the major promoter recognition subunit σ^{70} by σ^{S} factor in RNA polymerase complex. Hfq becomes mainly accumulated at the cytoplasm, dynamically shifting the transcripts' stabilities. The translation machinery is also modulated by the stationary-phase RMF (Ribosome Modulation Factor) that mediates direct 30S dimer contacts between 70S ribosome monomers at the P site (Azam et al., 2000) (Ishihama, 1999). Those translationaly incompetent 100S ribosomes may consist of inactive storage structures that remain available for future fast resumption of growth, once conditions change to favourable – ribosomal "hibernation" stage (Yoshida et al., 2002). Proteolysis by the ClpXP and ClpAP complexes appears to be mandatory to extended stationary-phase bacterial cultures viability, controlling the levels of growth–phase regulated proteins (Weichart et al., 2003).

The universal stress response regulator σS sigma-factor

Transition into stationary phase induces the formation of a core set of several proteins, members of specific stress-inducible regulons, elucidating why

stationary-phase cells are more resistant to several stresses (heat shock, H_2O_2, NaCl, alkylating agents, ethanol, acetone, toluene, deoxycholate, acidic or basic pH). Among those is the σ^S subunit of RNA polymerase, a major regulator of many stationary-phase-inducible genes, that controls several morphological and physiological processes in survival to prolonged starvation periods (Hengge-Aronis, 1996) (Loewen et al., 1998). A reduced growth rate results in increased *rpoS* transcription whereas high osmolarity, low temperature, acidic pH, and some late log-phase signals stimulate the translation of already present *rpoS* mRNA. In addition, carbon starvation, high osmolarity, acidic pH, and high temperature result in stabilization of σ^S protein, which otherwise is degraded (Hengge-Aronis, 2002) (Hengge-Aronis, 1996). Important cis-regulatory determinants as well as trans-acting regulatory factors involved at all levels of σ^S regulation have been identified: *rpoS* mRNA stability and translation are controlled by several proteins (Hfq, H-NS, HU, LeuO, CspC, CspE, cAMP-CRP complex and even DnaK) and by small regulatory RNAs (dsrA RNA and OxyR RNA). The molecular mechanisms of signal transduction that trigger the stationary-phase response and the induction of σ^S have yet to be completely elucidated, but seem to involve molecular signals as ppGpp, UDP-glucose, and homoserine lactone. Polyadenylation decreases the recognition of σ^S specific by the specific factor RssB (also designated by SprE (Carabetta et al., 2009)), essential for σ^S proteolysis by ClpXP protease. After translation, sigma S activity is still positively modulated by trehalose and glutamate (Phadtare and Inouye, 2001) (Hengge-Aronis, 1996) (Hengge-Aronis, 2002) (Loewen et al., 1998).

Sigma S controls the expression of genes that respond to general stresses, usually involved in the uptake and metabolism of aminoacids, sugars, iron and production of indole, among others. It also regulates several regulatory proteins, as ribosome associated protein RpsV (sra), the initiation factor IF-1 (infA) (Lacour

and Landini, 2004), the Dps protein (DNA-binding protein) and CsgD, homologous to the DNA binding domains of the LuxR family; and BolA.

FINELY TUNNED REGULATION OF *bolA*

bolA has been described as an *ftsZ* dependent gene, which is required to produce *bolA*-mediated round cells. By analysis of *bolA* open reading frame (ORF), it was showed that it encodes a protein with a predicted molecular weight of 13.5 kDa, transcribed in a clockwise direction on the *E. coli* chromosome. This gene is regulated by two different promoters, a P1 promoter, under the control of σ^S and an upstream P2 promoter, controlled by σ^D (Fig. 2). Moreover, *bolA* mRNA initiated 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). However, transcripts originated by P1 promoter are primarily detected when cells enter late-exponential phase of growth, beginning of stationary phase or upon imposition of stress conditions with a concomitant decrease of bolAp2 mRNA levels (Aldea et al., 1989). This observation gave rise to an hypothesis of promoter occlusion, stating that the physical interaction of the transcription machinery by σ^S at the *bolA*1p promoter, could interfere with the ability of RNA polymerase to either interact or proceed from *bolA*2p (Santos et al., 1999).

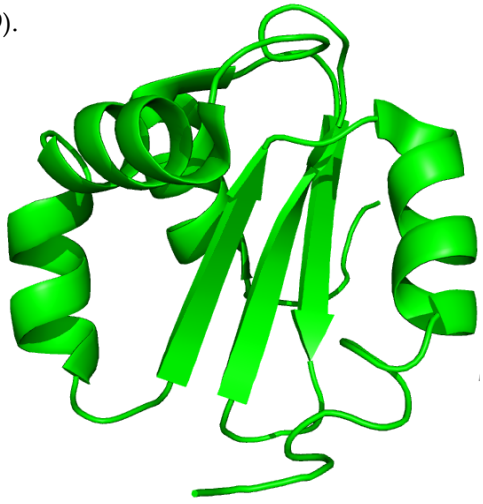


Figure 1. BolA protein structure. 2DHM PDB was taken from http://www.pdb.org/ and the cartoon picture created using *pymol* evaluation software.

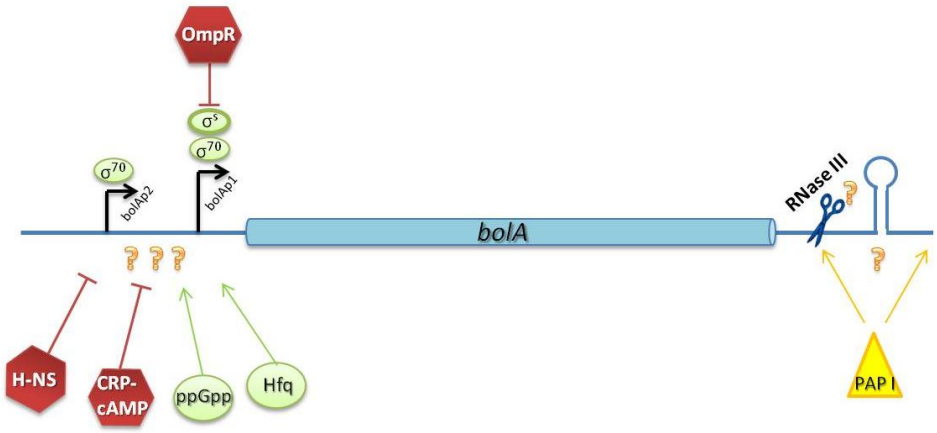


Figure 2. bolA1p (P1) promoter is mainly regulated by the σ^S transcription factor and induced in stationary phase and stress conditions. bolA2p (P2) promoter is σ^{70}-dependent and is constitutively expressed. OmpR, H-NS, and CRP-cAMP downregulate while ppGpp upregultes *bolA* transcription. Both ribonuclease III and Hfq stabilize *bolA1p* transcript. Question marks indicate regions of interaction or cut yet to be confirmed.

P1 promoter is growth phase and growth rate regulated and contains a gearbox element characteristic of several other structural genes, some of which are also induced in stationary phase. These type of promoters are induced inversely to the growth rate (Aldea et al., 1989). Additionally, it was also observed that the bolA P1 promoter, similarly to *rpoS* (encoding σ^S) can be downregulated by cAMP levels (Lange and Hengge-Aronis, 1991). At the transcriptional level, ribonuclease III has also an important role in the regulation of the expression of the *bolA* gene (Freire et al., 2006a; Santos et al., 1997). RNase III is a double-stranded endoribonuclease widely distributed among prokaryotic and eukaryotic organisms. Moreover, it is encoded by the *rnc* gene, and is active as a 52 kDa homodimer (Arraiano et al., 2010). RNase III is described as responsible for *rpoS* mRNA regulation, acting as modulator of σ^S levels in glucose starvation, ensuring σ^S normal levels of expression (Freire et al., 2006a; Santos et al., 1997). Since RNase III regulates σ^S levels, it indirectly regulates the levels of *bolA* mRNA. Another factor controlling *bolA* mRNA is polyadenylation, which inversely correlates with bacterial growth rate (Jasiecki and Wegrzyn, 2003), and 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 that *bolA*1p is negatively regulated *in vitro* by phosphor-OmpR (Yamamoto, 2000).

At the post-transcriptional level, under carbon starvation, RNase III is also involved in the *bolA*1p stability. The *bolA*1p mRNA is induced nine fold in a wt strain while in an *rnc* mutant strain is fourfold induced, showing a decrease in *bolA*1p RNA in the absence of RNase III (Freire et al., 2006a). It has been shown that the stationary phase messengers such as *rpoS* and *bolA* are polyadenylated (Cao and Sarkar, 1997). Therefore, polyadenylation probably interferes in their post-transcriptional control.

BolA, a stress response effector protein

Similarly to *rpoS*, *bolA* induced gene expression occurs at the transition to stationary phase related to the decrease in cells growth rate, but also in exponential phase after imposition of virtually any stress condition (Aldea et al., 1989; Lange and Hengge-Aronis, 1991; Santos et al., 1999).

In all stress trials developed, BolA was overexpressed and its cellular effects were noticeable, in spite of some differences. Sudden carbon starvation and increased osmolarity, respectively, resulted in about 17-fold and 22-fold increase in mRNA levels derived from *bolA1p* 1 h after stress imposition on exponential phase of growth (more than three and fourfold of what happened in stationary phase). A threefold increase of *bolA1p* mRNA levels is registered in response to both heat shock and acidic stress. Heat shock induction of *bolA1p* is immediate (maximum after 15 min), and mRNA levels reduction along time relate to significant increase in optical densities and moderate recovery in viabilities (possibly due to favoring elongation to division). The acid induction of *bolA1p* expression is more gradual, and while cells are able to divide, their growth rate never resumes the initial. In

contrast, the *bolA1p* mRNA gradual increase, up to eight times, 2 hours after the onset of H_2O_2 oxidative stress, and inhibits both growth and viabilities (Santos et al., 1999). *bolA1p* stress induction overrides the normal regulation imposed by growth rate, which is strictly the result of σ^S-directed transcription. In fact, there is differential dependence on σ^S for induction of *bolA1p* mRNA levels under different stress conditions, and certain basal stress response operate in the absence of σ^S. The stress-dependent activation of *bolA1p* should confer *E. coli* Gram-negative bacteria protection against a variety of stresses, based on the reduction of surface area exposed to damaging agents while decreasing the surface to volume ratio and promoting biofilm formation (Santos et al., 1999).

BolA "MORPHOGENETIC DETERMINISM"

BolA effects are pleiotropic, but a special interest goes to cell wall related mechanisms (Fig. 3), since bacteria morphology is profoundly affected by an increase in *bolA* levels (Fig. 4).

BolA can act over elongation, peptidoglycan synthesis and maturation or cell division mechanisms, or even regulating cytosqueletal elements.

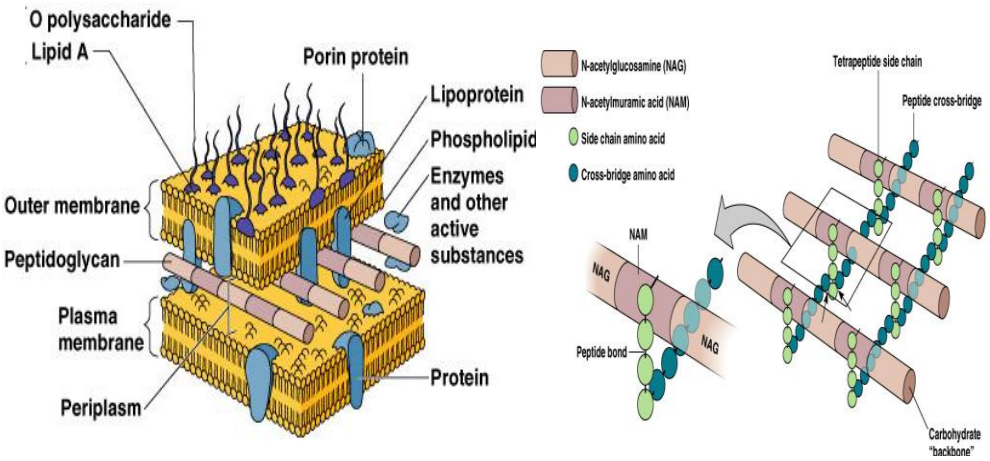


Figure 3. Gram-negative bacteria membrane and cell wall organization with particular incidence on the sacculus polymerization (right) taken from http://classes.midlandstech.edu/.

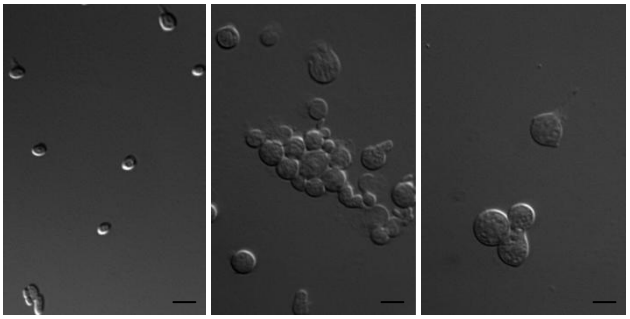


Figure 4. *Escherichia coli bolA* overexpression strain (MG1655 derived with pMAK580) morphologies due to increasing intracellular levels of BolA protein. Initially BolA induces regular sized spherical shapes, which enlarge as levels increase. The dark bars represent 5 µm

Peptidoglycan synthesis and properties

Peptidoglycan is a vital bacterial macromolecule, around 6 nm thick (Matias et al., 2003), that provides a structure that maintains cell shape, mediating interactions and regulating exchanges between the internal cellular media and their environment (Hughes et al., 1975; Vollmer et al., 2008). It constitutes the sacculus, that presents a flexibility due to the ability of changing cell length while maintaining the diameter virtually unaltered (van den Bogaart et al., 2007). Even though murein contains and maintains bacterial size and shape, those are not merely determined based on the chemical composition or structure of the peptidoglycan polymer, but depends also on cellular morphogenetic apparatus since restoration of murein in spheroplasts leads to the formation of round *sacculi* (Schwarz and Leutgeb, 1971; Weidel et al., 1960). Polar murein is not differentially composed from the lateral one, even though it is metabolically inert and significantly less deformable, eventually due to predominant alignment of the (more flexible) peptides in the direction of the long axis with the (more rigid) glycan strands perpendicularly arranged (Boulbitch et al., 2000). When septation was prevented, patches of stable murein were also observed at regular intervals, corresponding to future poles of filaments (Rothfield, 2003). Polar inert peptidoglycan influences outer membrane proteins mobility, the free movement of non-reacting protein species, and secretion generating asymmetries (den Blaauwen et al., 2008).

During exponential growth, there is a fast and progressive enrichment of the murein *sacculus* in newly synthesized trimers and dimers of lipoprotein-bound muropeptides at the expense of the respective monomers, at the tetra to tripeptide side chains and not at the oligosaccharide glycan chains of the peptidoglycan precursors (Burman and Park, 1983). Particularly in resting cells, and progressively when cultures are reaching stationary phase, murein globally changes its structure becoming hypercross-linked and richer in covalently bound lipoprotein, while reducing the mean length of the glycan chains. These modifications may provide extra resistance, hardening the damaging agents entering into cells, and the eventual peptidoglycan hydrolysis, although they might also complicate the cell wall metabolism and thus require structural changes to occur when in transition to active growth (Pisabarro et al., 1985).

Duplication of bacterial cells is quite demanding in terms of processes that need to occur in synchrony. Before septation, cells seem to require a minimum of previous lateral elongation, a process which demands not only membrane and cytosqueleton expansion and rearrangements, but also extension of the murein *sacculus* (den Blaauwen et al., 2008; Donachie and Begg, 1989; Grover et al., 1977). In fact, when rod cells are imposed spherical forms (using *rodA* or *pbpA* sensitive mutants) they maintain cell length and the ratio of DNA/mass, in a way that *cocci* increase both their volume and DNA contents four to six times, thus suggesting a minimum cell length for nucleoid separation (Donachie and Begg, 1989). The peptidoglycan extension occurs by addition and polymerization, into the pre-existent *sacculus*, of new alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) nucleotide sugar-linked – disaccharidepentapeptide units - precursor strands, that later on become interconnected by tetrapeptides (through β-1→4 bonds) derived from the MurNAc residues (Fig.3).

Polymerization of the peptidoglycan precursors is operated by transglycosylases – the High Molecular Weight (HMW) PBPs PBP1a, PBP1b, PBP1c, PBP2 and PBP3 - and cross-linking is catalysed by transpeptidases (Ghuysen, 1991; Holtje, 1998; Sauvage et al., 2008). At least the remodelling of the murein *sacullus* is mediated by carboxypeptidases (which remove the terminal D-ala residues from the pentapeptide) and endopeptidases (which hydrolyse the glycan chains' cross-links) and eventually hydrolases like amidases and LD-carboxypeptidases (Holtje, 1998; Nicholas et al., 2003; Priyadarshini et al., 2007; Templin, 2004).

Cell wall elongation progress

Bacterial elongation occurs by simultaneous incorporation of precursors in small number - since some of the components of the elongation machinery are scarce (20 to 40 molecules of PBP2 per cell) (Den Blaauwen et al., 2003; Spratt, 1975; Spratt, 1977). They locate along MreB filaments (interacting with MreC and D (Dye et al., 2005; Figge et al., 2004)) and FtsZ spirals (Daniel and Errington, 2003; den Blaauwen et al., 2008). Synthesis occurs by insertion of cross-linked glycan strands (Bertsche et al., 2005; Born et al., 2006; den Blaauwen et al., 2008) thus creating "mosaic structures made up of (…) all-new and all-old peptidoglycan" with fast turn-over (De Pedro et al., 2003a; den Blaauwen et al., 2008; Koch and De Pedro, 2006). Elongation may either be a continuous process along cell cycle or stall while PBP2 is relocated to the constriction site helping in the septation process, in a way that cell poles would be synthesized both by lateral and septal mode, given that its blockage inhibits peptidoglycan synthesis in a constant value (60%). Blocking PBP3 in turn reflects accordingly to the constriction processes: about 35%, periodically (Thibessard et al., 2002; Wientjes and Nanninga, 1991; Zapun et al., 2008).

The hierarchical, highly regulated and time controlled divisional process

Upon polymerization of the Z-ring (see below "*The bacterial cytosqueleton*"), several other proteins concur to the midcell contributing to the septation process. This is a very well defined hierarchic, constitutionally interdependent and time controlled event (Fig. 5).

Initially FtsZ filaments interact with FtsA (47KDa) in a short conserved C-terminal end site that overlaps the one of ZipA where they both modulate the protofilaments stability by tethering them to the membrane (Addinall and Lutkenhaus, 1996; den Blaauwen et al., 2008; Hale and de Boer, 1999; Lowe et al., 2004; Pichoff and Lutkenhaus, 2007). ZapA or YshA is other 10KDa non-essential protein that interacts with FtsZ rendering the filaments more stable and enhancing bundle (Gueiros-Filho and Losick, 2002). Specifically in high osmolarity conditions FtsZ-ring requires the interaction with the ABC transporter FtsE/X to be assembled (Corbin et al., 2007; Schmidt et al., 2004). S-adenosylmethionine transferase (SAM) is also essential to the divisome assembly, eventually due to the inability of FtsQ, W, I and/or N to locate properly at the Z-ring (Wang et al., 2005). FtsK is the first enzyme to be recruited, after the FtsZ ring is polymerized. It is a multifunctional and multidomain protein whose cytoplasmic C-terminal domain is involved in DNA segregation and N-terminal transmembrane domain is responsible for cell division (Bigot et al., 2007; Dorazi and Dewar, 2000). Although *ftsK* null mutants are compensated when FtsA or FtsQ are overexpressed, they tend to generate multiseptated filament cells suggesting an additional role in final closure of the newly formed poles (Geissler and Margolin, 2005). In addition, the deletion of *dacA* which encodes for PBP5 can also reverse to rods the filaments formed due to a single mutation in one of the transmembrane helices of *ftsK44*, while its increase induces the formation of oval structures sequencially contricted along the filament cells (Begg et al., 1995).

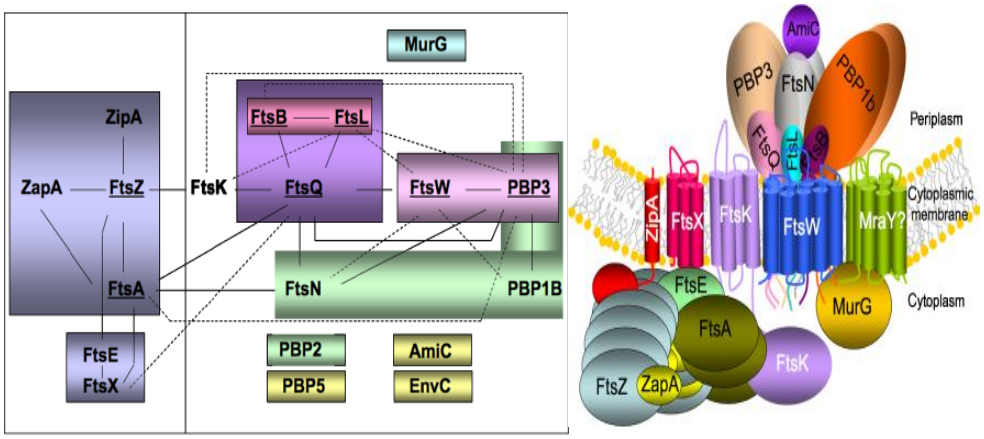


Figure 5. Schematic representation of the assembly of the components of the divisome. The boxed proteins represent subcomplexes: (a) the Z-ring with FtsZ-FtsA-ZipA-ZapA, which interacts with FtsE/X; (b) the subcomplex FtsQLB, which contains a heterodimer of FtsL and FtsB; (c) the subcomplex FtsW-PBP3; (d) PBP1B and FtsN interact with PBP3 and could be part of the subcomplex FtsW-PBP3. MurG, PBP2 and PBP5, AmiC and EnvC are located at the division and are part of the cell division machinery. Dashed lines: interaction detected using a two-hybrid system; Solid lines: interactions detected using different techniques. Copied from (den Blaauwen et al., 2008) by courtesy of Benoît Wolff.

FtsQ, L and B are membrane proteins bearing a small N-terminal intracellular region connected to the C-terminal periplasmic domain through a transmembrane helix (Chen et al., 2002; D'Ulisse et al., 2007; Scheffers et al., 2007; van den Ent et al., 2008). FtsQ interacts with PBP3, FtsW and FtsN (D'Ulisse et al., 2007; Di Lallo et al., 2003; Karimova et al., 2005) and is furthermore able to back recruit FtsK, making it a good candidate for the regulation of new pole synthesis (Goehring et al., 2005). FtsL and B complex is enough to recruit FtsW and PBP3 to the septasome. It thus seems that later division proteins group into complexes previously to their final arrangement, perhaps allowing to overcome eventual flaws and ensuring survival (Goehring et al., 2006). Similarly to the elongation machinery, these complexes are limited in numbers- there are about 20-40 FtsQ and 100 PBP3 molecules per cell (den Blaauwen et al., 2008).

PBP3 is targeted to the septa by its first 56 N-terminal amino acid residues shown to interact with FtsW, the first 70 with FtsQ, the periplasmic non-catalitic module with FtsL, apart from the C-terminal penicillin-binding transpeptidase activity module (Adam et al., 1997; Karimova et al., 2005; Piette et al., 2004). This protein is additionally responsible for the localization of PBP1b at the division site independently of its activity and the complex interacts with FtsN (Derouaux et al., 2008; Karimova et al., 2005). PBP1c then interacts with PBP3 and PBP1b, which also interacts with FtsW (Derouaux et al., 2008; Schiffer and Holtje, 1999). Finally, MipA, a structural scaffolding protein for murein, interacts with PBP1b and MltA, an outer membrane lipoprotein and lytic transglycosylase that hydrolyses murein (Lommatzsch et al., 1997); and MgtA, a monofunctional peptidoglycan glycosyltransferase that catalyzes glycan chain elongation of the bacterial cell wall, interacts with PBP3, FtsW, and FtsN (Derouaux et al., 2008). In terms of localization, all the PBP1 bifunctional transglycosylase-transpeptidase family of proteins seem to participate indifferently in the lateral and septal murein synthesis (Bertsche et al., 2006).

AmiC, a periplasmic N-acetylmuramyl-L-alanine amidase, and EnvC, a murein hydrolase, which locate at the Z-ring during constriction, are suggested to be responsible for final cleavage of the septal peptidoglycan, consequentially separating the daughter cells. At least AmiC requires FtsN for proper localization and the enzymatic performance (Bernhardt and de Boer, 2004); (Bernhardt and de Boer, 2003; Heidrich et al., 2001).

The peptidoglycan and some of the respective division machinery seem to be essential for bacterial cell growth even in cells known to have no cell wall like L-form-like *E. coli* and Chlamidial species (Joseleau-Petit et al., 2007; McCoy and Maurelli, 2006). Cell division is truly inseparable of cell wall synthesis at mid-cell/future poles (Joseleau-Petit et al., 2007). Besides the peptidoglycan and membrane, the outer membrane is also required to constrict and separate in order

to finalize the division from mother to daughter cells. For that to occur all five of the Tol-Pal system proteins concur, transiently accumulating from dispersed to the mid-cell during constriction, requiring FtsN for such location (Gerding et al., 2007).

Cell wall maturation/plasticity/protection by Low Molecular Weight PBPs

The probable trigger for the septation mode of peptidoglycan synthesis is the increase in the amount of tripeptides in the *sacculus*, through an increase of DD-carboxypeptidation by PBP5 and/or PBP6. Spherical cells may arise from this increase simply due to the weakening of the structure in a way that the cell cannot endure a rod shape. Supporting this is the fluctuation of the levels of carboxypeptidase activity, preakly increasing at the constriction period of the cell cycle. An increase in carboxypeptidase activities and decrease in pentapeptide precursor levels of murein could stimulate cross-linking by PBP3 instead of PBP2 as seen immediately before and during cell division (Mirelman et al., 1977; Mirelman et al., 1978). Indeed, deletion of PBP5 produces the accumulation of muropeptides with pentapeptide side-chains; and neither BolA absence or overexpression show changes in murein ultrastructure from the parental strains (Santos et al., 2002). On another hand, increased levels of PBP5 or PBP6 leads to similar phenotype as increased amount of tripeptides through addition of D-cycloserine. Actually, the overexpression of either PBP5 or PBP6, but not PBP4, can reverse the effects of a specific temperature-sensitive allele of PBP3 (Begg et al., 1990), and PBP5 overexpression converts rods into spheres (Markiewicz et al., 1982). In fact, the location of inert murein to the polar caps seems to be dependent on PBP5. Upon blocking cell division, *dacA* defective *Escherichia coli* strains exhibit a branching phenotype where peptidoglycan regions of constrained synthesis, turnover, and protein mobility appear throughout the side-walls of the cells, and the poles often split in half due to the emergence of active synthesis at the pole

sites (de Pedro et al., 2003b). Additionally, the loss of PBP5, similarly to the increase in β–lactamases expression, creates defects in biofilm formation and bacterial adhesion properties (Gallant et al., 2005).

Although a lot of studies have been done, the low molecular weight (LMW) PBPs remain a mystery. They are in highest cellular copy number PBPs (Dougherty et al., 1996), nevertheless single or global deletion leads to viable similarly growing cultures and only PBP5 of all eight LMW PBPs has a visible phenotype (Denome et al., 1999). PBP4 seems to act as a limited substrate endopeptidase (Korat et al., 1991; Meberg et al., 2004; Mottl and Keck, 1991) and its overexpression induces AmpC (Mottl et al., 1992)

BolA regulation over the DD-carboxypeptidases PBP5 and PBP6

PBP5 and PBP6 share 65% identity and the peptidoglycan remodeling type of activity, although the second presents about 4 times less specific activity (Amanuma and Strominger, 1980). In addition, overexpression of either of them can reverse the effects of a specific temperature-sensitive allele of PBP3, again showing to perform similar functions *in vivo* (Begg et al., 1990). However, this two proteins do not functionally overlap on the basis of their carboxypeptidase activities, or on their penicillin binding abilities (Ghosh and Young, 2003); and overexpression of one or the other leads to completely different results: high levels of PBP5 originate spheres out of rods (Markiewicz et al., 1982) and the same is not seen for PBP6 (van der Linden et al., 1992). On the other hand, the latter was suggested to stabilize by cross-linking (van der Linden et al., 1992) the peptidoglycan of nongrowing cells (Glauner and Holtje, 1990) and aminoacid starved cells (Goodell and Tomasz, 1980), in analogy to the *Bacillus subtilis* sporulation-specific PBP5a (PBP5*) (Buchanan and Ling, 1992; Todd et al., 1985). An increase of the *dacA* (PBP5) and *dacC* (PBP6) mRNA levels has been established when *bolA* levels are increased(Santos et al., 2002) and a strain where

bolA gene was deleted seemed to show a decrease in PBP5 or PBP6 protein levels (Aldea et al., 1989).

The bacterial cytosqueleton

Even though bacteria lack organelles, the bacterial cytosqueleton emerges as an absolute necessity, since all functions have to be performed in a limited and accelerated time scale. Basic cellular functions are ensured efficiently by gathering enzymes that participate in specific traits, in a way that mimics compartmentalization, thus enhancing products formation (Moller-Jensen and Lowe, 2005). Cytosqueleton molecules and fibres are thus not only essential for conferring cells the mechanical support and resistance, create orientation/polar discrimination and determine and maintain their shape, but also may be the means to locate and connect different molecules in their functional dynamics. The most obvious processes in which cytosqueleton elements are tightly implicated are DNA segregation and cell division. The cytosqueleton molecules that have been described so far, are being unravelled to present a flexible, highly dynamical helical pattern of polymerization, when not limited by space constraints (Moller-Jensen and Lowe, 2005).

FtsZ is a 37-43 kDa protein and has around 5000 copies per cell (Pla et al., 1991). It is a microtubule-like protein forming essential filaments for cell division, localizing at the midcell for the cytokinetic Z-ring formation during septation where 30-40% of this molecules form straight filaments hydrolysing GTP. They can also form lesser pronounced curved helical protofilaments hydrolysing GDP (Lu et al., 2000). In fact, FtsZ polymer is highly dynamic presenting a turn-over time constant of about 30 sec and an exchange rate between the membrane polymerized form and the cytoplasmic pool of 8-10 sec (Stricker et al., 2002) (Anderson et al., 2004). A conformational change model has been proposed by Erickson, 1997 according to which GTP hydrolysis of the FtsZ filaments to the

GDP form and the consequent transition from straight to curved structures would induce the polymer to bend and thus constrict together with the bound membrane (Erickson, 1997). The FtsZ-ring assembles from about 30 protofilaments subunits overlapped in ~120 nm fibre structures (Chen and Erickson, 2005; Li et al., 2007) and is responsible for membrane constriction when cells are dividing and as a trigger for division proteins assembly orderly: FtsZ, FtsA/ZipA, FtsK, FtsQ, FtsL, FtsB, FtsW, FtsI (PBP3), and FtsN into the septasome or divisome (den Blaauwen et al., 2008). This sequential and interdependent process is also negatively regulated by several different elements such as the 19 kDa SulA protein that responds to DNA damage binding the T7-loop catalytic pocket preventing polymerization until all DNA is repaired; Min CDE(or DivIVA in *Bacillus subtilis*) system that oscillates from pole to pole, every 20-50 sec, thus preventing septation to occur at the poles (Corbin et al., 2002; Raskin and de Boer, 1999); nucleoid occlusion by Noc non-specific DNA binding protein that prevents septation to cut DNA; and the *Bacillus subtilis* non-essential inner-membrane 62kDa protein EzrA that inhibits aberrant ring formation (Levin et al., 1999; Moller-Jensen and Lowe, 2005).

In *E. coli,* the *mreB* gene is in an operon composed by 3 genes: *mreB*, *mreC* and *mreD*. This operon is expressed as monocistronic *mreB* and polycistronic *mreB*CD mRNA (Freire et al., 2009; Wachi et al., 2006). *E.coli* MreB, as *Baccilus subtilis* Mbl demonstrated to have a turn-over rate of 8 min (Carballido-Lopez and Errington, 2003); MreB and ParM form the actin-like cytosqueleton in bacteria. The first two molecules develop into 51/55 Aº spaced protofilaments that distort into non-polar helices spiralling in networks inside the cells, immediately below the membranes (Jones et al., 2001; Lowe et al., 2004). These molecules would be enough to confer the shape, determine the dimensions, and promote the rigidity of the respective cells, by their own resistance and/or continuous assembly against the membranes (Erickson, 2001; M Doi, 1988). Even though in *B. sutilis* Mlc and MreB

complement to control width and linear axis dimensions respectively, MreB is alone in its task to maintain cell shape determination in *E. coli* (Jones et al., 2001). MreB protein requires the membrane and periplasmic dimer MreC, the integral membrane MreD and RodA proteins (but not PBP2) to localize correctly (Esue et al., 2005; Karczmarek et al., 2007; Kruse et al., 2005; van den Ent et al., 2001; van den Ent et al., 2006). Moreover MreB as Mlc actively segregate the chromosomes (Kruse et al., 2003), eventually through the interaction with SetB (a sugar membrane transport protein) (Espeli et al., 2003; Liu JY, 1999), localizing origins of replication towards opposite cell halves (Gitai Z, 2004 ; Soufo and Graumann, 2003) [like ParM which requires ATP and is regulated by ParR (Moller-Jensen et al., 2002; Moller-Jensen et al., 2003)] and generate cell polarity by spacially arranging polar proteins (Gitai Z, 2004). MreB seems to achieve equal cell partition by duplication and segregation to opposite poles of a doublet ring structure formed in both halves of dividing cells in a previous and independent even of cytokinesis(Vats and Rothfield, 2007). MreB seems to be recruited to induce proper placement of the murein biosynthetic machinery and may be transiently assembled or work as a permanent scaffold for elongation (Osborn and Rothfield, 2007). Finally, crescentin is a recently studied intermediate filaments-like bacterial protein that polymerizes into 10nm thick filaments, specifically localizes in the concave faced cytoplasm of Caulobacter (Ausmees, 2006).

Apart from the commonly considered cytosqueleton elements, another class has been determined of "cytomotive elements" designated by WACA filaments – Walker A cytosqueletal ATPases – proteins that self-polymerize but simultaneously adhere to membrane surfaces or DNA, hydrolyzing ATP, and promoting cellular positioning (Lowe and Amos, 2009). MinD binds and polymerizes on membranes aided by the MinE activator to increase the MinC inhibitor in the poles; ParABS is a type I plasmid partitioning system similar to

the actin-like type II, ParMRC for which ParA is represents the WACA; Soj is the chromosomal version of ParA binding unspecifically to DNA, moving from the condensed part of the nucleoid to the opposite side, aided by the activator Spo0J; ParF forms filaments in the absence of surfaces together with the adaptor ParG (Lowe and Amos, 2009).

BolA and the cytosqueleton elements

As mentioned above, to produce round cells when overexpressed, *bolA* requires the presence of an active *ftsZ* gene product (Aldea et al., 1988). A second important factor in *E. coli* cell division and shape determination is MreB. MreB forms helical structures underneath the cytoplasmic membranes responsible for maintaining the typical rod shape of *E. coli* (Doi et al., 1988). A defect in *mreB* gene causes a partially similar phenotype to *bolA* overexpression, round cells. The similar phenotype caused by *bolA* lead to a recent study where it was showed that *bolA* acts as a regulator of *mreB* (Freire et al., 2009). Strong evidences were observed regarding *bolA* preventing cellular elongation when certain key factors were inhibited, like the PBP2. MreB polymers forming the cytosqueleton are visible when *bolA* is present at basal levels in the cell. However, when *bolA* is overexpressed, the filaments are detected but spread in the round shape cell, showing the effect of *bolA* in the filaments spatial organization. That may be the means by which BolA induces the loss of *E. coli* rod shape to sphere (Fig. 6). Moreover, *bolA* was proven to be a transcription factor directly interacting with *mreBCD* promoters. Upon *bolA* overexpression *mreBCD* operon transcription shifts to about 64% of its normal expression (Freire et al., 2009).

Figure 6. Schematic representation of the shape change induced by BolA along the cultures growth. In the transition to stationary phase BolA levels increase significantly and mreB is decreased in turn. As both of this protein levels are modulated rod cells become spherical.

PERMEABILITY MODULATION AND BIOFILM DEVELOPMENT AS STRESS ADAPTATION MECHANISMS

Biofilms consist of complex highly organized and dynamic bacterial communities composed of specialized individuals even when issued from a single colony. This explains why they sometimes show altruistic behaviour that would not be expected in single cell individuals (Bayles, 2007). When facing challenges, damaged cells induce autolysis [a bacterial analogue mechanism to the Programmed Cell Death in eukaryotes (Lewis, 2000)] thus eliminating competitors for nutrient availability and releasing their intracellular contents, providing a new pool of macromolecules to their surroundings. Apart from the importance of DNA in creating the adherent structure that characterizes biofilms (Whitchurch et al., 2002), it was acknowledged to be orderly positioned within those structures revealing an organization in the bacterial death (again similarly to the apoptosis in eukaryotes) within those communities (Allesen-Holm et al., 2006). A minimum amount of cell death (less than 1% within the first 24 hours) provides the necessary amount of nucleic acids for the population stabilization (Rice et al., 2007).

Biofilm development is a phenomena with major medical and economic consequences (Bayles, 2007). Biofilm formation is usually associated with altered environments, increasing the resistance of cells to extreme conditions, thus enduring toxic substances, like antibiotics and host immune defence products

(Vieira 2004). As stated above, *bolA* is a stress induced gene in *E. coli*, changing the morphology to shorter and rounder cells (Freire et al., 2009; Lange and Hengge-Aronis, 1991). Surprisingly, when biofilm formation was analysed in a *bolA* mutant strain, it was observed a decrease in biofilm formation during stress conditions. Moreover, particularly in nutrient limitation conditions, and oxidative stress, the presence of *bolA* increased biofilm thickness around fivefold compared to the *bolA* mutant strain (Fig. 7), strengthening the role of *bolA* in the cell defence mechanisms against stress (Vieira 2004).

The bacteria outer membrane (OM) is a very important barrier against mechanical or chemical stresses and external factors that bacteria are exposed to. The Gram-negative OM is composed by lipid bilayers with little permeability for hydrophilic solutes. In its composition, there are channel forming proteins allowing the passage of nutrients and expulsion of waste. OmpF and OmpC are the two major constituents of *E. coli* OM, being OmpF a more permeable porin than OmpC. When facing hostile conditions, bacteria produce more OmpC and repress OmpF expression (Freire et al., 2006b; Nikaido et al., 1983). This difference in the balance of expression of the different OM channels can be related to BolA. When *bolA* is highly expressed, cells are more resistant to detergents and antibiotics, process which relates to the composition of the OM. This may help to explain how *bolA* potentially contributes to the protection against external injuries and relates to its induction of biofilm formation (Freire et al., 2006b).

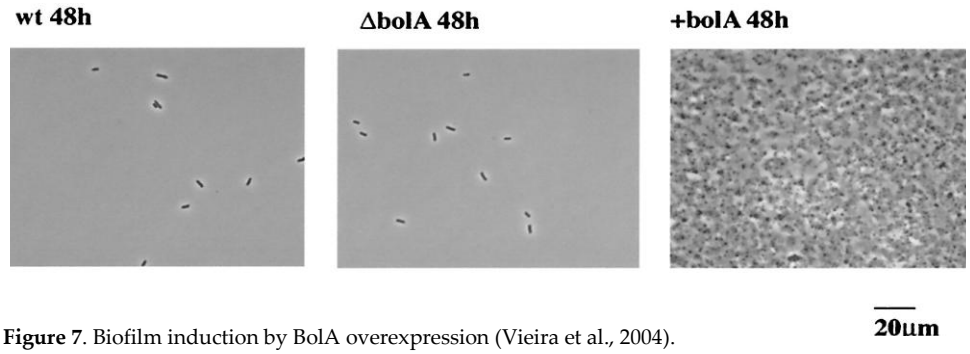


Figure 7. Biofilm induction by BolA overexpression (Vieira et al., 2004).

SYNOPSIS AND FUTURE PERSPECTIVES

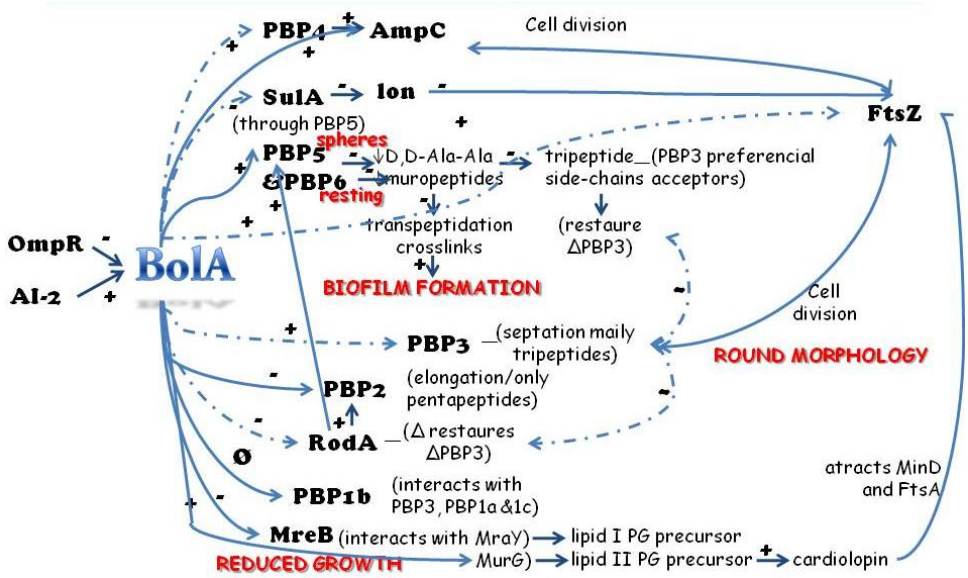


Figure 8. BolA molecular interaction synopsis board. Continuous arrows indicate published results; dashed lines purpose regulatory effects not yet evaluated but inferred by related effects. Most "BolA effectors" relate to cell wall metabolism/maintenance/control systems.

BolA homologues

BolA-like proteins are widely conserved from prokaryotes to eukaryotes (Huynen et al., 2005). They seem to be involved in cell proliferation or cell-cycle regulation, but their molecular function is still unknown. The overall topology of a mouse BolA-like protein is similar to the class II KH fold, except for the absence of the usually well conserved GXXG loop (Kasai et al., 2004). NMR structures have been made public in the NCBI site for PDBs. Interestingly, all the conserved residues in the BolA-like proteins are assembled on the one side of the protein (Kasai et al., 2004). The three-dimensional structures of BolA-like proteins from *Mus musculus* and *Xanthomonas campestris pv. campestris* show similarities to nucleic acid-binding proteins (Chin et al., 2005; Kasai et al., 2004). *Escherichia coli* protein also exhibits a helix-turn-helix motif that should correspond to the DNA-binding

domain, through which it interacts and transcriptionally regulates different genes (Aldea et al., 1989; Freire et al., 2009; Guinote et al., 2010). It can already be found described amongst several other transcriptional factors and sigma-factors networks of interaction in *E. coli* and even other non-redundant bacterial genomes (Martinez-Antonio et al., 2008) (Fig. 8).

This *E. coli* 13.5 kDa morphogene protein, encoded from the 10 min region of the genetic map, induces size reduction and spherical morphology on rod shaped bacteria. Expressed under stress conditions, reducing surface area of cells, inducing biofilm formation, changing the outer membrane properties, accessibility and sensitivity towards detergents and antibiotics and modulating permeability, eventually through modulation of OmpF/OmpC balance (Aldea et al., 1989) (Vieira et al., 2004) (Freire et al., 2006b), *bolA* is suggested to be involved in protection and resistance mechanisms promotion.

This nucleic acid binding transcriptional regulator protein, seems to be implicated in the switching between cell elongation and septation during the cell cycle. This action seems to occur at least through the transcriptional activation of cell wall hydrolytic DD-carboxypeptidases Penicillin-Binding Proteins *PBP5* and *PBP6* and possibly *AmpC*, concerted with the downregulation of the *mreB* cytosqueleton element (Freire et al., 2009; Santos et al., 2002).

The *Schizosaccharomyces pombe* UV-inducible *uvi31+* gene which encodes a 12 kDa protein with 57% amino acid sequence similarity to *Escherichia coli* BolA protein, has its expression cell cycle–regulated and growth phase–dependent (Kim et al., 1997). Furthermore, the *bolA* yeast homologue *uvi31+* who accelerates spore germination, decreases proliferation rate, enhances cell size in vegetative growth, controls the correct septum formation and cytokinesis, confers UV resistance and is eventually responsible for the control of cell division, especially on resumption from cell cycle arrest (Kim et al., 2002). Interestingly, while the deletion mutant exhibited various anomalies in septation and cytokinesis the overexpression of

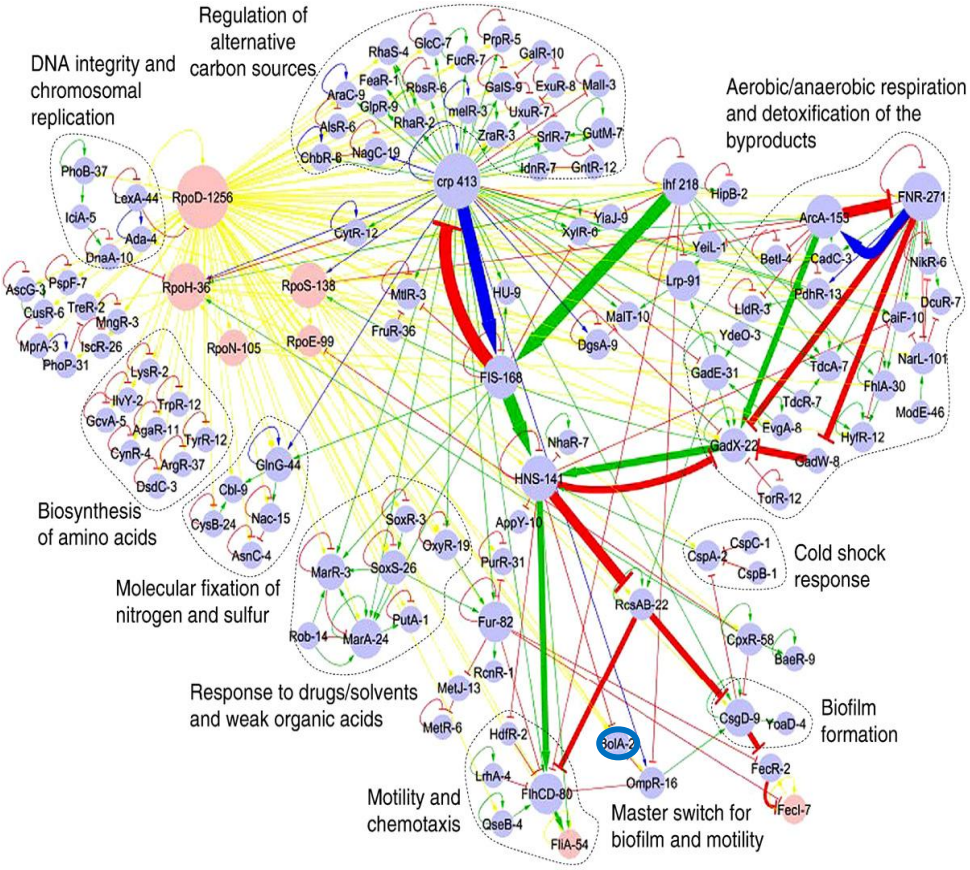


Figure 8. Core transcriptional regulatory network of E. coli. Blue and pink nodes represent genes encoding for TFs and sigma factors, respectively; each node label is accompanied with its connectivity showing the number of regulatory targets. Edges represent cross-regulatory interactions (green for activation, red for repression, blue for dual interactions and yellow for sigma transcription), whereas loops represent transcriptional autoregulations. Specific subnetworks, such as the one associated with the regulation of carbon sources, are delineated with dashed lines to distinguish different regulatory modules. This figure was generated using Cytoscape and published at (Martinez-Antonio et al., 2008).

uvi31+ did not produce significant changes in cell proliferation or division (Kim et al., 2002).

Alternatively, BolA has been predicted to function as a reductase through interaction with the prokaryotic genomes neighbours mono-thiol glutaredoxins (which would provide it reducing equivalents to the evolutionarily conserved cysteines' loss) and thus be responsible for reducing organic peroxides (like it's

structurally more closely related OsmC) (Huynen et al., 2005). In addition, strong phylogenetic connection is observed for genes encoding BolA and monothiol glutathioneredoxins as they are genome co-occurrent: either both BolA-like proteins and CGFS-type Grx are present or absent in most organisms (Couturier et al., 2009; Huynen et al., 2005). Accordingly, genome-wide yeast two-hybrid assays and proteome-wide FLAG- and TAP-tag affinity purification studies have identified a physical interaction between cytosolic monothiol Grxs and BolA-like proteins in *E. coli*, yeast and *Drosophila melanogaster* (Butland et al., 2005; Giot et al., 2003; Ho et al., 2002; Ito et al., 2000; Krogan et al., 2006). Recently yeast Grx3 and Grx4 were determined to directly interact with the BolA-like protein Fra2 (Kumanovics et al., 2008) and to be involved in the intracellular Fe signaling, through the formation of Fe-S cluster binding regulatory complex where the [2Fe-2S]-containing heterodimers show different cluster coordination to the [2Fe-2S]-bridged Grx3 or Grx4 homodimers reconstituted in the absence of Fra2 (Li et al., 2009). Initial characterization of a *bolA* homologue in *Pseudomonas fluorescens* has suggested a link to sulphur metabolism, but the deletion phenotype could only be complemented through simultaneous addition of the 2 downstream (out of the 7) genes of the operon, encoding a putative sulphur reductase and disulphide isomerase, respectively. Thus, more is needed to understand the BolA-like proteins functions in this organism (Koch and Nybroe, 2006).

Moreover, not only *Escherichia coli* but several Proteobacterial genomes, have been determined to harbor two genes encoding BolA type proteins (Tatusov et al., 2001). However, functional studies have been carried out only for the *E. coli* BolA protein. In this work, we have analyzed the *yrbA Escherichia coli* homologue, which was renamed of *ibaG*, for "induced by acid gene" (see http://www.ncbi.nlm.nih.gov/protein/ NP_417657.2, and Chapter 4 of this Dissertation) strengthening the idea of stress endurance by BolA-type proteins.

Function on persistence

In the past century bacterial infections were the major reason of premature death. With vaccination, antibiotics appearance and the development of new generation synthetic drugs prognosis for most of the microbial infections strongly inverted from certain death. But apart from mechanisms that genetically define a microorganism's ability to evade a particular antibiotics action, insights have arose into the aptitude microorganisms present of avoiding death due to random multi-external aggressions just by changing physiology or metabolism. Bacteria have been recognized, since the middle of the XXth century, to have the ability to "tolerate" or "persist" enduring strong environmental pressures, like antibiotics, in the absence of the genomic resistances, eventually through dormancy, without dividing (Bigger, 1944). This tolerance phenomenon, only termed in the 70s, does not occur due to specific antidrug mechanisms; instead, the ability to escape the lethality induced by the metabolism corrupters seems to rely on the "absence" of active metabolism in the cells – "if persisters are dormant and have little or no cell-wall synthesis, translation, or topoisomerase activity, when the antibiotics bind to their molecular targets they are nevertheless unable to corrupt their function (...) at the price of non-proliferation"(Lewis, 2007) (Fig.9).

It is not regarded as the fittest but instead an altruistic behaviour where cells take longer to resume growth (1 to 1,5 hours more) but can propagate their genetic background by "escaping" different kinds of lethal factors (Balaban et al., 2004).

Cells in culture do not increase their level of resistance to lethal substances, in fact, bulk cells are highly susceptible to antibiotics and speedily killed when exposed; nevertheless a minor persister multidrug resistant subpopulation is able to evade those effects, and repopulate after drug clearance, in this way favouring chronic infection. The ability to form persister cell varies along the growth curve: it is reduced in the initial steps but increases until stationary phase cells to about 1% of the population. This dependence on the bacterial physiological state was

confirmed by the inability to create persisters when cultures were diluted four times only reaching exponential phase of growth (Keren et al., 2004). Quorum sensing mechanisms did not seem to concur to this, since the use of spent growth medium (enriched of QS signalling molecules) did not lead to a persister cells enrichment (Lewis, 2007). This dynamics seems to rely on the inherent phenotypic heterogeneity of the bacterial populations, which may be dependent on "persister" protein levels accumulation, elicited by different environmental factors that affect the death process.

Pursuit of genes responsible for persistence has proven misleading due to the overlapping functions of different genes/proteins, avoiding the detection in single gene deletion libraries (Baba et al., 2006; Hansen et al., 2008).

Persister genes detected by deletion are generally global regulators – *dnaJ* and *dnaK* (chaperones), *apaH* (diadenosine tetraphosphatase), *surA* (peptidyl-prolyl cis-trans isomerase), *fis* and *hns* (global regulators), *hnr* (response regulator of RpoS), *dksA* (transcriptional regulator of rRNA transcription), *ygfA* (5-formyl-tetrahydrofolate cyclo-ligase), and *yigB* (flavin mononucleotide [FMN] phosphatase), the latter two not being global regulators but depleting cells in folate and FMN pools - once more, strengthening the idea of redundancy. Some genes that might contribute are *rmf*, a stationary phase inhibitor of translaction (Yoshida et al., 2002), *sulA*, an inhibitor of septation (Unoson and Wagner, 2008) and strong inducer of persister formation in a SOS pathway dependent way (Dorr et al.), *carB*, a central enzyme for pyrimidine and arginine biosynthetic pathways (Beaumont et al., 2009), and toxin-antitoxin (TA) loci like *relBE*, *dinJ*, *yafQ*, *ygiU*, *mazEF*, *tisAB* (Christensen and Gerdes, 2003; Christensen et al., 2003; Lewis), and *hipA(B)*, that stops cell growth conferring drug tolerance (Correia et al., 2006). When considering the TA modules, persistence can be communicated among populations by plasmid transfer.

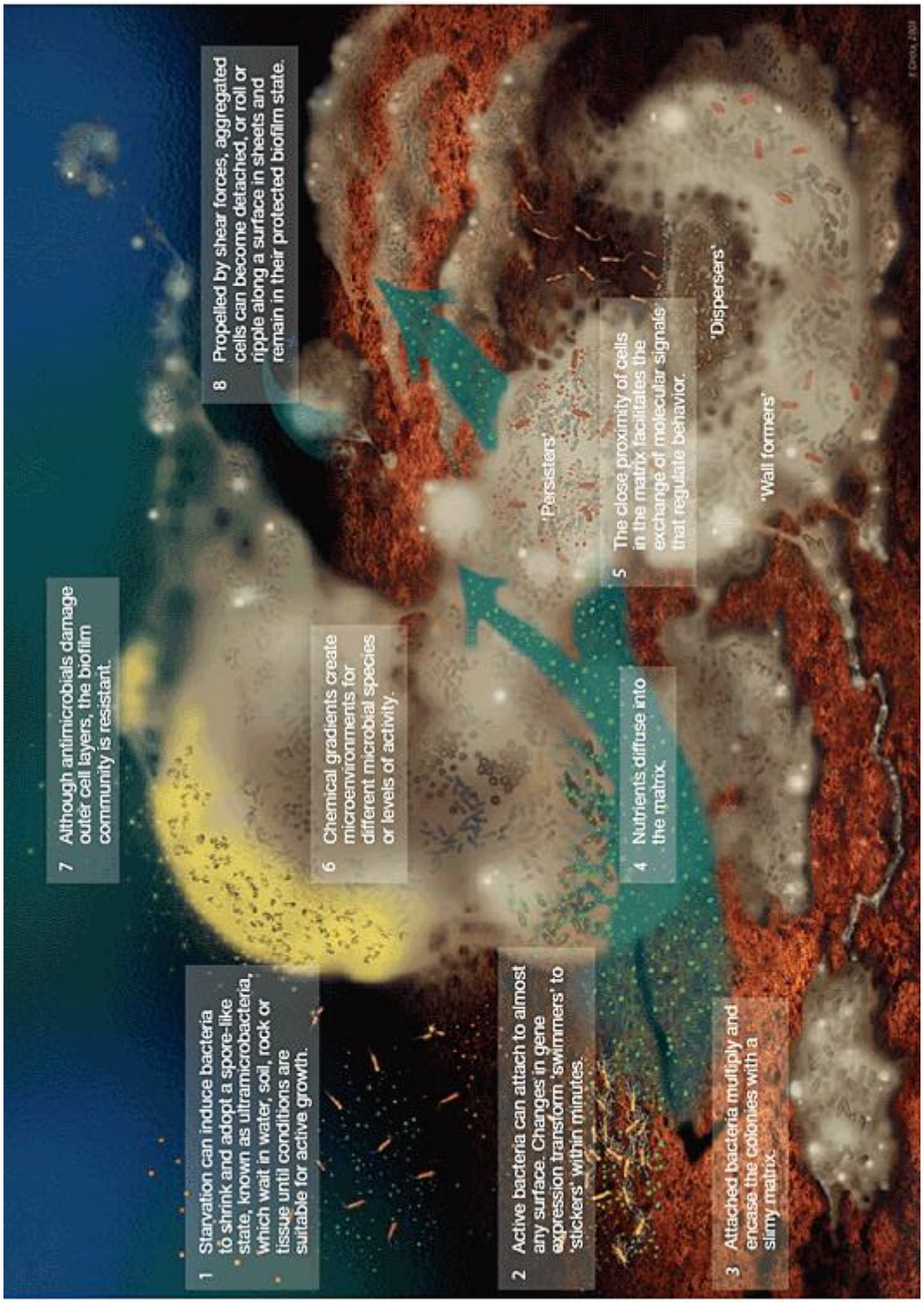

1 Starvation can induce bacteria to shrink and adopt a spore-like state, known as ultramicrobacteria, which wait in water, soil, rock or tissue until conditions are suitable for active growth.
2 Active bacteria can attach to almost any surface. Changes in gene expression transform 'swimmers' to 'stickers' within minutes.
3 Attached bacteria multiply and encase the colonies with a slimy matrix.
4 Nutrients diffuse into the matrix.
5 The close proximity of cells in the matrix facilitates the exchange of molecular signals that regulate behavior.
6 Chemical gradients create microenvironments for different microbial species or levels of activity.
7 Although antimicrobials damage outer cell layers, the biofilm community is resistant.
8 Propelled by shear forces, aggregated cells can become detached, or roll or ripple along a surface in sheets and remain in their protected biofilm state.
'Persisters'
'Wall formers'
'Dispersers'

Figure 9. Mechanism of persister development and characteristics taken form http://img.medscape.com/.

This dormant state may additionally explain the inability to grow most of the wild microbial populations – in the absence of certain recognized elements from their natural habitat, cells defend stalling growth and multiplication waiting for more familiar conditions (Lewis, 2007).

Another line of study, probably corresponding to the same mechanism, only analysed in a different perspective, lead to the conclusion that *Escherichia coli* cells become nonculturable or sterile upon starvation or growth arrest, this is, they lost their ability to reproduce in (nutrient agar) plates as a result of nutrient depletion, low temperatures, high pressure, changes in pH or salinity (Colwell and Grimes, 2000), as well as by induction of reduced peptidase activity due to mutations or addition of inhibitors of protein synthesis, while remaining individually structured and organized. Two alternative programming theories were proposed to explain such occurence: the formation of viable but nonculturable cells (VBNC) that originate intact dormant differentiated matured cells that become insensitive to the environment until it becomes favourable and only then resume activity - a process that would resemble spores differentiation awaiting for regrowth; or nonculturable senescent cells that have accumulated to much (free radical induced) damage leading to the production of aberrant proteins that become susceptible for oxidative attack and begin a programmed cell death, that will result in their fatality (Nystrom, 2003b; Nystrom, 2003c).

Stasis induced cross-protection strongly relies on the expression of several genes involved in the resistance against a number of different stresses (Hengge-Aronis, 1993; Matin, 1991). σ^S is the master regulator for stasis survival and stress resistance and this sigma factor reveals an antagonistic pleiotropy with the rate of

reproduction, trade-off that is due to the competition with σ^{70} for the RNA polymerase, for which it requires the presence of ppGpp, both for production and activity, probably priming the RNA polymerase for competitiveness (Gentry et al., 1993; Kvint et al., 2000; Lange et al., 1995; Nystrom, 2003a). σ^{S} (Dukan and Nystrom, 1998; Dukan and Nystrom, 1999; Hengge-Aronis, 1993; Yasuda et al., 1999); σ^{E} (Testerman et al., 2002), σ^{32} (Nystrom, 1999), the ArcA regulon [that controls the rate of reserves catabolism in stationary phase (Iuchi and Lin, 1991), helping cells protect against self-inflicted oxidative damage (Nystrom et al., 1996)], and the OxyR global regulator (Vlamis-Gardikas et al., 2002) are induced when less favourable growth conditions are imposed to cultures. Peptidase activity provides the aminoacids as subtrates for the required protein synthesis (Matin, 1991; Reeve et al., 1984a; Reeve et al., 1984b).

The TA loci also support the theory of self-preservation/mechanism to avoid deterioration since they do not in fact kill the cells, only impose a bacteriostatic condition, where cells become locked in a G_0-like state, which can be completely reversed. When external (& internal) conditions improve, ppGpp levels decrease and stringent response is abolished. As such, Lon and ClpP proteases reduce their presence in the cells, discontinuing the unnecessary degradation of proteins. Among those, the cognate antitoxins for the toxins that induce the growth arrest are left to bind their targets, and thus allow translation to resume its progress, general biosynthesis to restart, and cells to regain culturability (Nystrom, 2003c).

On the other hand, aging has been evaluated and actually accounted for in microorganisms, even *E. coli*. It was expected to occur in unicellular eukaryotes like budding yeast where cell division is assymmetric, creating a bigger mother cell able to divide until 24 times, with the last ones considerably slower and leading to granular and/or lysing cells (Jazwinski, 2002). Also *Caulobacter crescentus*, a bacterium that also divides asymmetrically leading to an adhesive

anchored stalk, and a smaller motile "swarmer" cell that eventually differentiates into a stalk, exhibits a degeneration process where stalk division takes twice the time after 100 generations (Ackermann et al., 2003). The passage through a swarmer stage apparently seems to rejuvenate the cells and reset the negative effects of mutations that cause earlier aging in mother cells (Ackermann et al., 2007). Surprisingly, a similar effect could be determined for *E. coli*. Although they divide symmetrically with a non-conservative dispersal of the cytoplasmic material, therefore supposedly dividing equally the damaged constituents, cell poles and DNA strands should not be considered of the same age (Nystrom, 2003a). Cells with two generation old poles were determined to grow about 2% slower then cells with one generation old poles, and this effects were additive with every generation (Stewart et al., 2005). Older poles would thus be working like aged and damaged material dumps (Stephens, 2005), perhaps as a strategy in symmetrical partitioning - those old poles seem to be inert, therefore they should not react so much to the accumulation of cellular waste, whose damage is thus prevented to be propagated into the biosynthetically active dynamic structures. Nevertheless, *E. coli* cells lack a mandatory aging process, since old macromolecules are quickly diluted due to the fast turnover of all cell components when the environment supports growth and proliferation (Nystrom, 2002).

Here we propose a unifying theory where Gram-negative cells strongly confronted with aggressive environments would initially change their metabolism reducing it to a minimum amount, condensate their DNA in order to protect it from eventual aggressions, translate chaperones and chaperorines to protect vital proteins, induce protein degradation of porins to reduce exchanges with the surroundings and other superfluous proteins to create pools of aminoacids, providing substrates for future metabolism reset, condensate

ribosomes to avoid further error-prone translation, induce the reduction of external surface changing form rods to spheres reducing the surface-volume ratio, differentiate surface membrane asymmetries protecting areas or extrude certain polymers into developing biofilms as protective barriers against the injuries, and other protection mechanisms. This initial response would be simultaneous with the formation of persister or viable but nonculturable cells, according to both studies definitions. After the long term growth arrest, with continuous aggression and accumulation of internal damages, cell death would be a fatality. The controled cell deaths would be important for the population survival as an all. Namely, some cell's deaths would lead to a reduction in the environmental pressure due to less resources competition. Moreover, they would provide some nutrients release and media renewal, protection molecules available from internal agregant/chelant components, and adhesion products for the formation of biofilms apart from constituting a physical barrier against the environmental toxics. The part of the population slowly accumulating injuries and entering into the programmed cell death would provide means for the others to survive in an altruist population instead of individual strategy. This point of view would reflect an analogue mechanism to apoptosis where cells initially react to avoid death with several molecules interacting and communicating, to finally reach the decision of life or death according to the internal prevailing situation. When there is too much injuries accumulated and the demands in recreating cellular homeostasis are high, the cell death and substitution by an all new structure formation (by cellular duplication) becomes more beneficial in terms of the population/species.

BolA protein may fit in between one or several of mechanisms just uncovered. It has been determined to change bacterial shape, and simultaneously confer resistance against large antibiotic molecules and detergents, by reducing

permeability, changing the outer membrane porins equilibrium, some peptidoglycan modification enzymes like the DD-carboxypeptidases PBP5 and PBP6 , which cross-link the murein, namely increasing its resistance against host protection lysozyme injuries, and strongly inducing biofilm development. This pleiotropic effector protein has recently been demonstrated to act as a transcriptional regulator, what accounts for such global cell phenotypes. Nevertheless, its action as a nucleic acid binding regulator and the targets determined do not completely explain the cell physiological behavior upon BolA expression. This global phenotype has several common elements with the persistence/tolerance/senescence features, partially responsible for the chronic diseases emergence and microorganism's multitude of stresses resistance. Molecular knowledge should be obtained to evaluate these hypotheses.

REFERENCES

Ackermann, M., S.C. Stearns, and U. Jenal. 2003. Senescence in a bacterium with asymmetric division. Science 300:1920.

Ackermann, M., A. Schauerte, S.C. Stearns, and U. Jenal. 2007. Experimental evolution of aging in a bacterium. BMC Evol Biol 7:126.

Adam, M., C. Fraipont, N. Rhazi, M. Nguyen-Disteche, B. Lakaye, J.M. Frere, B. Devreese, J. Van Beeumen, Y. van Heijenoort, J. van Heijenoort, and J.M. Ghuysen. 1997. The bimodular G57-V577 polypeptide chain of the class B penicillin-binding protein 3 of Escherichia coli catalyzes peptide bond formation from thiolesters and does not catalyze glycan chain polymerization from the lipid II intermediate. J Bacteriol 179:6005-9.

Addinall, S.G., and J. Lutkenhaus. 1996. FtsZ-spirals and -arcs determine the shape of the invaginating septa in some mutants of Escherichia coli. Mol Microbiol 22:231-7.

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 9:3787-94.

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 170:5169-76.

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 8:3923-31.

Ali Azam, T., A. Iwata, A. Nishimura, S. Ueda, and A. Ishihama. 1999. Growth phase-dependent variation in protein composition of the Escherichia coli nucleoid. J Bacteriol 181:6361-70.

Allesen-Holm, M., K.B. Barken, L. Yang, M. Klausen, J.S. Webb, S. Kjelleberg, S. Molin, M. Givskov, and T. Tolker-Nielsen. 2006. A characterization of DNA release in Pseudomonas aeruginosa cultures and biofilms. Mol Microbiol 59:1114-28.

Amanuma, H., and J.L. Strominger. 1980. Purification and properties of penicillin-binding proteins 5 and 6 from Escherichia coli membranes. J Biol Chem 255:11173-80.

Anderson, D.E., F.J. Gueiros-Filho, and H.P. Erickson. 2004. Assembly dynamics of FtsZ rings in Bacillus subtilis and Escherichia coli and effects of FtsZ-regulating proteins. J Bacteriol 186:5775-81.

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.

Ausmees, N. 2006. Intermediate filament-like cytosqueleton of Caulobacter crescentus. J Mol Microbiol Biotechnol 11:152-8.

Azam, T.A., S. Hiraga, and A. Ishihama. 2000. Two types of localization of the DNA-binding proteins within the Escherichia coli nucleoid. Genes Cells 5:613-26.

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. Mol Syst Biol 2:2006 0008.

Balaban, N.Q., J. Merrin, R. Chait, L. Kowalik, and S. Leibler. 2004. Bacterial persistence as a phenotypic switch. Science 305:1622-5.

Bayles, K.W. 2007. The biological role of death and lysis in biofilm development. Nat Rev Microbiol 5:721-6.

Beaumont, H.J., J. Gallie, C. Kost, G.C. Ferguson, and P.B. Rainey. 2009. Experimental evolution of bet hedging. Nature 462:90-3.

Begg, K.J., S.J. Dewar, and W.D. Donachie. 1995. A new Escherichia coli cell division gene, ftsK. J Bacteriol 177:6211-22.

Begg, K.J., A. Takasuga, D.H. Edwards, S.J. Dewar, B.G. Spratt, H. Adachi, T. Ohta, H. Matsuzawa, and W.D. Donachie. 1990. The balance between different peptidoglycan precursors determines whether Escherichia coli cells will elongate or divide. J Bacteriol 172:6697-703.

Bernhardt, T.G., and P.A. de Boer. 2003. The Escherichia coli amidase AmiC is a periplasmic septal ring component exported via the twin-arginine transport pathway. Mol Microbiol 48:1171-82.

Bernhardt, T.G., and P.A. de Boer. 2004. Screening for synthetic lethal mutants in Escherichia coli and identification of EnvC (YibP) as a periplasmic septal ring factor with murein hydrolase activity. Mol Microbiol 52:1255-69.

Bertsche, U., E. Breukink, T. Kast, and W. Vollmer. 2005. In vitro murein peptidoglycan synthesis by dimers of the bifunctional transglycosylase-transpeptidase PBP1B from Escherichia coli. J Biol Chem 280:38096-101.

Bertsche, U., T. Kast, B. Wolf, C. Fraipont, M.E. Aarsman, K. Kannenberg, M. von Rechenberg, M. Nguyen-Disteche, T. den Blaauwen, J.V. Holtje, and W. Vollmer. 2006. Interaction between two murein (peptidoglycan) synthases, PBP3 and PBP1B, in Escherichia coli. Mol Microbiol 61:675-90.

Bigger, J. 1944. Treatment of staphylococcal infections with penicillin by intermittent sterilization The Lancet 244 497-500.

Bigot, S., V. Sivanathan, C. Possoz, F.X. Barre, and F. Cornet. 2007. FtsK, a literate chromosome segregation machine. Mol Microbiol 64:1434-41.

Born, P., E. Breukink, and W. Vollmer. 2006. In vitro synthesis of cross-linked murein and its attachment to sacculi by PBP1A from Escherichia coli. J Biol Chem 281:26985-93.

Boulbitch, A., B. Quinn, and D. Pink. 2000. Elasticity of the rod-shaped gram-negative eubacteria. Phys Rev Lett 85:5246-9.

Buchanan, C.E., and M.L. Ling. 1992. Isolation and sequence analysis of dacB, which encodes a sporulation-specific penicillin-binding protein in Bacillus subtilis. J Bacteriol 174:1717-25.

Burman, L.G., and J.T. Park. 1983. Changes in the composition of Escherichia coli murein as it ages during exponential growth. J Bacteriol 155:447-53.

Butland, G., J.M. Peregrin-Alvarez, J. Li, W. Yang, X. Yang, V. Canadien, A. Starostine, D. Richards, B. Beattie, N. Krogan, M. Davey, J. Parkinson, J. Greenblatt, and A. Emili. 2005. Interaction network containing conserved and essential protein complexes in Escherichia coli. Nature 433:531-7.

Cao, G.J., and N. Sarkar. 1997. Stationary phase-specific mRNAs in Escherichia coli are polyadenylated. Biochem Biophys Res Commun 239:46-50.

Carabetta, V.J., B.K. Mohanty, S.R. Kushner, and T.J. Silhavy. 2009. The response regulator SprE (RssB) modulates polyadenylation and mRNA stability in Escherichia coli. J Bacteriol 191:6812-21.

Carballido-Lopez, R. 2006. Orchestrating bacterial cell morphogenesis. Mol Microbiol 60:815-9.

Carballido-Lopez, R., and J. Errington. 2003. The bacterial cytosqueleton: in vivo dynamics of the actin-like protein Mbl of Bacillus subtilis. Dev Cell 4:19-28.

Chen, J.C., M. Minev, and J. Beckwith. 2002. Analysis of ftsQ mutant alleles in Escherichia coli: complementation, septal localization, and recruitment of downstream cell division proteins. J Bacteriol 184:695-705.

Chen, Y., and H.P. Erickson. 2005. Rapid in vitro assembly dynamics and subunit turnover of FtsZ demonstrated by fluorescence resonance energy transfer. J Biol Chem 280:22549-54.

Chin, K.H., F.Y. Lin, Y.C. Hu, K.H. Sze, P.C. Lyu, and S.H. Chou. 2005. NMR structure note--solution structure of a bacterial BolA-like protein XC975 from a plant pathogen Xanthomonas campestris pv. campestris. J Biomol NMR 31:167-72.

Christensen, S.K., and K. Gerdes. 2003. RelE toxins from bacteria and Archaea cleave mRNAs on translating ribosomes, which are rescued by tmRNA. Mol Microbiol 48:1389-400.

Christensen, S.K., K. Pedersen, F.G. Hansen, and K. Gerdes. 2003. Toxin-antitoxin loci as stress-response-elements: ChpAK/MazF and ChpBK cleave translated RNAs and are counteracted by tmRNA. J Mol Biol 332:809-19.

Colwell, R.R., and D.J. Grimes. 2000. Nonculturable microorganisms in the environment. Semantics and Strategies, ASM Press:1-6.

Corbin, B.D., X.C. Yu, and W. Margolin. 2002. Exploring intracellular space: function of the Min system in round-shaped Escherichia coli. Embo J 21:1998-2008.

Corbin, B.D., Y. Wang, T.K. Beuria, and W. Margolin. 2007. Interaction between cell division proteins FtsE and FtsZ. J Bacteriol 189:3026-35.

Correia, F.F., A. D'Onofrio, T. Rejtar, L. Li, B.L. Karger, K. Makarova, E.V. Koonin, and K. Lewis. 2006. Kinase activity of overexpressed HipA is required for growth arrest and multidrug tolerance in Escherichia coli. J Bacteriol 188:8360-7.

Couturier, J., J.P. Jacquot, and N. Rouhier. 2009. Evolution and diversity of glutaredoxins in photosynthetic organisms. Cell Mol Life Sci 66:2539-57.

D'Ulisse, V., M. Fagioli, P. Ghelardini, and L. Paolozzi. 2007. Three functional subdomains of the Escherichia coli FtsQ protein are involved in its interaction with the other division proteins. Microbiology 153:124-38.

Daniel, R.A., and J. Errington. 2003. Control of cell morphogenesis in bacteria: two distinct ways to make a rod-shaped cell. Cell 113:767-76.

De Pedro, M.A., H. Schwarz, and A.L. Koch. 2003a. Patchiness of murein insertion into the sidewall of Escherichia coli. Microbiology 149:1753-61.

de Pedro, M.A., K.D. Young, J.V. Holtje, and H. Schwarz. 2003b. Branching of Escherichia coli cells arises from multiple sites of inert peptidoglycan. J Bacteriol 185:1147-52.

Den Blaauwen, T., M.E. Aarsman, N.O. Vischer, and N. Nanninga. 2003. Penicillin-binding protein PBP2 of Escherichia coli localizes preferentially

in the lateral wall and at mid-cell in comparison with the old cell pole. Mol Microbiol 47:539-47.

den Blaauwen, T., M.A. de Pedro, M. Nguyen-Disteche, and J.A. Ayala. 2008. Morphogenesis of rod-shaped sacculi. FEMS Microbiol Rev 32:321-44.

Denome, S.A., P.K. Elf, T.A. Henderson, D.E. Nelson, and K.D. Young. 1999. Escherichia coli mutants lacking all possible combinations of eight penicillin binding proteins: viability, characteristics, and implications for peptidoglycan synthesis. J Bacteriol 181:3981-93.

Derouaux, A., B. Wolf, C. Fraipont, E. Breukink, M. Nguyen-Disteche, and M. Terrak. 2008. The monofunctional glycosyltransferase of Escherichia coli localizes to the cell division site and interacts with penicillin-binding protein 3, FtsW, and FtsN. J Bacteriol 190:1831-4.

Di Lallo, G., M. Fagioli, D. Barionovi, P. Ghelardini, and L. Paolozzi. 2003. Use of a two-hybrid assay to study the assembly of a complex multicomponent protein machinery: bacterial septosome differentiation. Microbiology 149:3353-9.

Doi, M., M. Wachi, F. Ishino, S. Tomioka, M. Ito, Y. Sakagami, A. Suzuki, and M. Matsuhashi. 1988. Determinations of the DNA sequence of the mreB gene and of the gene products of the mre region that function in formation of the rod shape of Escherichia coli cells. J Bacteriol 170:4619-24.

Donachie, W.D., and K.J. Begg. 1989. Cell length, nucleoid separation, and cell division of rod-shaped and spherical cells of Escherichia coli. J Bacteriol 171:4633-9.

Dorazi, R., and S.J. Dewar. 2000. Membrane topology of the N-terminus of the Escherichia coli FtsK division protein. FEBS Lett 478:13-8.

Dorr, T., M. Vulic, and K. Lewis. Ciprofloxacin causes persister formation by inducing the TisB toxin in Escherichia coli. PLoS Biol 8:e1000317.

Dougherty, T.J., K. Kennedy, R.E. Kessler, and M.J. Pucci. 1996. Direct quantitation of the number of individual penicillin-binding proteins per cell in Escherichia coli. J Bacteriol 178:6110-5.

Dukan, S., and T. Nystrom. 1998. Bacterial senescence: stasis results in increased and differential oxidation of cytoplasmic proteins leading to developmental induction of the heat shock regulon. Genes Dev 12:3431-41.

Dukan, S., and T. Nystrom. 1999. Oxidative stress defense and deterioration of growth-arrested Escherichia coli cells. J Biol Chem 274:26027-32.

Dye, N.A., Z. Pincus, J.A. Theriot, L. Shapiro, and Z. Gitai. 2005. Two independent spiral structures control cell shape in Caulobacter. Proc Natl Acad Sci U S A 102:18608-13.

Erickson, H.P. 1997. FtsZ, a tubulin homologue in prokaryote cell division. Trends Cell Biol 7:362-7.

Erickson, H.P. 2001. Cytosqueleton. Evolution in bacteria. Nature 413:30.

Espeli, O., P. Nurse, C. Levine, C. Lee, and K.J. Marians. 2003. SetB: an integral membrane protein that affects chromosome segregation in Escherichia coli. Mol Microbiol 50:495-509.

Esue, O., M. Cordero, D. Wirtz, and Y. Tseng. 2005. The assembly of MreB, a prokaryotic homolog of actin. J Biol Chem 280:2628-35.

Figge, R.M., A.V. Divakaruni, and J.W. Gober. 2004. MreB, the cell shape-determining bacterial actin homologue, co-ordinates cell wall morphogenesis in Caulobacter crescentus. Mol Microbiol 51:1321-32.

Freire, P., R.N. Moreira, and C.M. Arraiano. 2009. BolA inhibits cell elongation and regulates MreB expression levels. J Mol Biol 385:1345-51.

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 88:341-6.

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 260:106-11.

Frenkiel-Krispin, D., S. Levin-Zaidman, E. Shimoni, S.G. Wolf, E.J. Wachtel, T. Arad, S.E. Finkel, R. Kolter, and A. Minsky. 2001. Regulated phase transitions of bacterial chromatin: a non-enzymatic pathway for generic DNA protection. Embo J 20:1184-91.

Gallant, C.V., C. Daniels, J.M. Leung, A.S. Ghosh, K.D. Young, L.P. Kotra, and L.L. Burrows. 2005. Common beta-lactamases inhibit bacterial biofilm formation. Mol Microbiol 58:1012-24.

Geissler, B., and W. Margolin. 2005. Evidence for functional overlap among multiple bacterial cell division proteins: compensating for the loss of FtsK. Mol Microbiol 58:596-612.

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. J Bacteriol 175:7982-9.

Gerding, M.A., Y. Ogata, N.D. Pecora, H. Niki, and P.A. de Boer. 2007. The trans-envelope Tol-Pal complex is part of the cell division machinery and required for proper outer-membrane invagination during cell constriction in E. coli. Mol Microbiol 63:1008-25.

Ghosh, A.S., and K.D. Young. 2003. Sequences near the active site in chimeric penicillin binding proteins 5 and 6 affect uniform morphology of Escherichia coli. J Bacteriol 185:2178-86.

Ghuysen, J.M. 1991. Serine beta-lactamases and penicillin-binding proteins. Annu Rev Microbiol 45:37-67.

Giot, L., J.S. Bader, C. Brouwer, A. Chaudhuri, B. Kuang, Y. Li, Y.L. Hao, C.E. Ooi, B. Godwin, E. Vitols, G. Vijayadamodar, P. Pochart, H. Machineni, M.

Welsh, Y. Kong, B. Zerhusen, R. Malcolm, Z. Varrone, A. Collis, M. Minto, S. Burgess, L. McDaniel, E. Stimpson, F. Spriggs, J. Williams, K. Neurath, N. Ioime, M. Agee, E. Voss, K. Furtak, R. Renzulli, N. Aanensen, S. Carrolla, E. Bickelhaupt, Y. Lazovatsky, A. DaSilva, J. Zhong, C.A. Stanyon, R.L. Finley, Jr., K.P. White, M. Braverman, T. Jarvie, S. Gold, M. Leach, J. Knight, R.A. Shimkets, M.P. McKenna, J. Chant, and J.M. Rothberg. 2003. A protein interaction map of Drosophila melanogaster. Science 302:1727-36.

Gitai Z, D.N., Shapiro L. 2004 An actin-like gene can determine cell polarity in bacteria. Proc Natl Acad Sci U S A. 101:8643-8.

Glauner, B., and J.V. Holtje. 1990. Growth pattern of the murein sacculus of Escherichia coli. J Biol Chem 265:18988-96.

Goehring, N.W., F. Gueiros-Filho, and J. Beckwith. 2005. Premature targeting of a cell division protein to midcell allows dissection of divisome assembly in Escherichia coli. Genes Dev 19:127-37.

Goehring, N.W., M.D. Gonzalez, and J. Beckwith. 2006. Premature targeting of cell division proteins to midcell reveals hierarchies of protein interactions involved in divisome assembly. Mol Microbiol 61:33-45.

Goodell, W., and A. Tomasz. 1980. Alteration of Escherichia coli murein during amino acid starvation. J Bacteriol 144:1009-16.

Grover, N.B., C.L. Woldringh, A. Zaritsky, and R.F. Rosenberger. 1977. Elongation of rod-shaped bacteria. J Theor Biol 67:181-93.

Gueiros-Filho, F.J., and R. Losick. 2002. A widely conserved bacterial cell division protein that promotes assembly of the tubulin-like protein FtsZ. Genes Dev 16:2544-56.

Guinote, I.B., R.G. Matos, P. Freire, and C.M. Arraiano. 2010. BolA affects growth and binds to the promoters of Penicillin-Binding Proteins 5 and 6 regulating their expression. Journal of Microbiology and Biotechnology:*in press*.

Hale, C.A., and P.A. de Boer. 1999. Recruitment of ZipA to the septal ring of Escherichia coli is dependent on FtsZ and independent of FtsA. J Bacteriol 181:167-76.

Halsey, T.A., A. Vazquez-Torres, D.J. Gravdahl, F.C. Fang, and S.J. Libby. 2004. The ferritin-like Dps protein is required for Salmonella enterica serovar Typhimurium oxidative stress resistance and virulence. Infect Immun 72:1155-8.

Hansen, S., K. Lewis, and M. Vulic. 2008. Role of global regulators and nucleotide metabolism in antibiotic tolerance in Escherichia coli. Antimicrob Agents Chemother 52:2718-26.

Heidrich, C., M.F. Templin, A. Ursinus, M. Merdanovic, J. Berger, H. Schwarz, M.A. de Pedro, and J.V. Holtje. 2001. Involvement of N-acetylmuramyl-L-

alanine amidases in cell separation and antibiotic-induced autolysis of Escherichia coli. Mol Microbiol 41:167-78.

Hengge-Aronis, R. 1993. Survival of hunger and stress: the role of rpoS in early stationary phase gene regulation in E. coli. Cell 72:165-8.

Hengge-Aronis, R. 1996. Back to log phase: sigma S as a global regulator in the osmotic control of gene expression in Escherichia coli. Mol Microbiol 21:887-93.

Hengge-Aronis, R. 1999. Interplay of global regulators and cell physiology in the general stress response of Escherichia coli. Curr Opin Microbiol 2:148-52.

Hengge-Aronis, R. 2002. Signal transduction and regulatory mechanisms involved in control of the sigma(S) (RpoS) subunit of RNA polymerase. Microbiol Mol Biol Rev 66:373-95, table of contents.

Ho, Y., A. Gruhler, A. Heilbut, G.D. Bader, L. Moore, S.L. Adams, A. Millar, P. Taylor, K. Bennett, K. Boutilier, L. Yang, C. Wolting, I. Donaldson, S. Schandorff, J. Shewnarane, M. Vo, J. Taggart, M. Goudreault, B. Muskat, C. Alfarano, D. Dewar, Z. Lin, K. Michalickova, A.R. Willems, H. Sassi, P.A. Nielsen, K.J. Rasmussen, J.R. Andersen, L.E. Johansen, L.H. Hansen, H. Jespersen, A. Podtelejnikov, E. Nielsen, J. Crawford, V. Poulsen, B.D. Sorensen, J. Matthiesen, R.C. Hendrickson, F. Gleeson, T. Pawson, M.F. Moran, D. Durocher, M. Mann, C.W. Hogue, D. Figeys, and M. Tyers. 2002. Systematic identification of protein complexes in Saccharomyces cerevisiae by mass spectrometry. Nature 415:180-3.

Holtje, J.V. 1998. Growth of the stress-bearing and shape-maintaining murein sacculus of Escherichia coli. Microbiol Mol Biol Rev 62:181-203.

Hughes, R.C., P.F. Thurman, and E. Stokes. 1975. Estimates of the porosity of Bacillus licheniformis and Bacillus subtilis cell walls. Z Immunitatsforsch Exp Klin Immunol 149:126-35.

Huynen, M.A., C.A. Spronk, T. Gabaldon, and B. Snel. 2005. Combining data from genomes, Y2H and 3D structure indicates that BolA is a reductase interacting with a glutaredoxin. FEBS Lett 579:591-6.

Ishihama, A. 1999. Modulation of the nucleoid, the transcription apparatus, and the translation machinery in bacteria for stationary phase survival. Genes Cells 4:135-43.

Ito, T., K. Tashiro, S. Muta, R. Ozawa, T. Chiba, M. Nishizawa, K. Yamamoto, S. Kuhara, and Y. Sakaki. 2000. Toward a protein-protein interaction map of the budding yeast: A comprehensive system to examine two-hybrid interactions in all possible combinations between the yeast proteins. Proc Natl Acad Sci U S A 97:1143-7.

Iuchi, S., and E.C. Lin. 1991. Adaptation of Escherichia coli to respiratory conditions: regulation of gene expression. Cell 66:5-7.

Jasiecki, J., and G. Wegrzyn. 2003. Growth-rate dependent RNA polyadenylation in Escherichia coli. EMBO Rep 4:172-7.

Jazwinski, S.M. 2002. Growing old: metabolic control and yeast aging. Annu Rev Microbiol 56:769-92.

Jones, L.J., R. Carballido-Lopez, and J. Errington. 2001. Control of cell shape in bacteria: helical, actin-like filaments in Bacillus subtilis. Cell 104:913-22.

Joseleau-Petit, D., J.C. Liebart, J.A. Ayala, and R. D'Ari. 2007. Unstable Escherichia coli L forms revisited: growth requires peptidoglycan synthesis. J Bacteriol 189:6512-20.

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.

Karimova, G., N. Dautin, and D. Ladant. 2005. Interaction network among Escherichia coli membrane proteins involved in cell division as revealed by bacterial two-hybrid analysis. J Bacteriol 187:2233-43.

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 13:545-8.

Keren, I., N. Kaldalu, A. Spoering, Y. Wang, and K. Lewis. 2004. Persister cells and tolerance to antimicrobials. FEMS Microbiol Lett 230:13-8.

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 14:425-30.

Kim, S.H., M. Kim, J.K. Lee, M.J. Kim, Y.H. Jin, R.H. Seong, S.H. Hong, C.O. Joe, and S.D. Park. 1997. Identification and expression of uvi31+, a UV-inducible gene from Schizosaccharomyces pombe. Environ Mol Mutagen 30:72-81.

Koch, A.L., and M.A. De Pedro. 2006. Partition of old murein in small patches over the entire wall of E. coli cells forced to grow as a coccoid. Curr Microbiol 52:249-53.

Koch, B., and O. Nybroe. 2006. Initial characterization of a bolA homologue from Pseudomonas fluorescens indicates different roles for BolA-like proteins in P. fluorescens and Escherichia coli. FEMS Microbiol Lett 262:48-56.

Korat, B., H. Mottl, and W. Keck. 1991. Penicillin-binding protein 4 of Escherichia coli: molecular cloning of the dacB gene, controlled overexpression, and alterations in murein composition. Mol Microbiol 5:675-84.

Krogan, N.J., G. Cagney, H. Yu, G. Zhong, X. Guo, A. Ignatchenko, J. Li, S. Pu, N. Datta, A.P. Tikuisis, T. Punna, J.M. Peregrin-Alvarez, M. Shales, X. Zhang, M. Davey, M.D. Robinson, A. Paccanaro, J.E. Bray, A. Sheung, B. Beattie, D.P. Richards, V. Canadien, A. Lalev, F. Mena, P. Wong, A. Starostine, M.M. Canete, J. Vlasblom, S. Wu, C. Orsi, S.R. Collins, S. Chandran, R. Haw, J.J. Rilstone, K. Gandi, N.J. Thompson, G. Musso, P. St Onge, S. Ghanny, M.H. Lam, G. Butland, A.M. Altaf-Ul, S. Kanaya, A. Shilatifard, E. O'Shea, J.S. Weissman, C.J. Ingles, T.R. Hughes, J. Parkinson, M. Gerstein, S.J. Wodak, A. Emili, and J.F. Greenblatt. 2006. Global landscape of protein complexes in the yeast Saccharomyces cerevisiae. Nature 440:637-43.

Kruse, T., J. Bork-Jensen, and K. Gerdes. 2005. The morphogenetic MreBCD proteins of Escherichia coli form an essential membrane-bound complex. Mol Microbiol 55:78-89.

Kruse, T., J. Moller-Jensen, A. Lobner-Olesen, and K. Gerdes. 2003. Dysfunctional MreB inhibits chromosome segregation in Escherichia coli. Embo J 22:5283-92.

Kumanovics, A., O.S. Chen, L. Li, D. Bagley, E.M. Adkins, H. Lin, N.N. Dingra, C.E. Outten, G. Keller, D. Winge, D.M. Ward, and J. Kaplan. 2008. Identification of FRA1 and FRA2 as genes involved in regulating the yeast iron regulon in response to decreased mitochondrial iron-sulfur cluster synthesis. J Biol Chem 283:10276-86.

Kvint, K., A. Farewell, and T. Nystrom. 2000. RpoS-dependent promoters require guanosine tetraphosphate for induction even in the presence of high levels of sigma(s). J Biol Chem 275:14795-8.

Lacour, S., and P. Landini. 2004. SigmaS-dependent gene expression at the onset of stationary phase in Escherichia coli: function of sigmaS-dependent genes and identification of their promoter sequences. J Bacteriol 186:7186-95.

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 173:4474-81.

Lange, R., D. Fischer, and R. Hengge-Aronis. 1995. Identification of transcriptional start sites and the role of ppGpp in the expression of rpoS, the structural gene for the sigma S subunit of RNA polymerase in Escherichia coli. J Bacteriol 177:4676-80.

Levin, P.A., I.G. Kurtser, and A.D. Grossman. 1999. Identification and characterization of a negative regulator of FtsZ ring formation in Bacillus subtilis. Proc Natl Acad Sci U S A 96:9642-7.

Lewis, K. Persister Cells. Annu Rev Microbiol.

Lewis, K. 2000. Programmed death in bacteria. Microbiol Mol Biol Rev 64:503-14.

Lewis, K. 2007. Persister cells, dormancy and infectious disease. Nat Rev Microbiol 5:48-56.

Li, H., D.T. Mapolelo, N.N. Dingra, S.G. Naik, N.S. Lees, B.M. Hoffman, P.J. Riggs-Gelasco, B.H. Huynh, M.K. Johnson, and C.E. Outten. 2009. The yeast iron regulatory proteins Grx3/4 and Fra2 form heterodimeric complexes containing a [2Fe-2S] cluster with cysteinyl and histidyl ligation. Biochemistry 48:9569-81.

Li, Z., M.J. Trimble, Y.V. Brun, and G.J. Jensen. 2007. The structure of FtsZ filaments in vivo suggests a force-generating role in cell division. Embo J 26:4694-708.

Liu JY, M.P., Willard J, Olson ER. 1999. Functional and biochemical characterization of Escherichia coli sugar efflux transporters. J Biol Chem. 274:22977-84.

Loewen, P.C., B. Hu, J. Strutinsky, and R. Sparling. 1998. Regulation in the rpoS regulon of Escherichia coli. Can J Microbiol 44:707-17.

Lommatzsch, J., M.F. Templin, A.R. Kraft, W. Vollmer, and J.V. Holtje. 1997. Outer membrane localization of murein hydrolases: MltA, a third lipoprotein lytic transglycosylase in Escherichia coli. J Bacteriol 179:5465-70.

Lowe, J., and L.A. Amos. 2009. Evolution of cytomotive filaments: the cytosqueleton from prokaryotes to eukaryotes. Int J Biochem Cell Biol 41:323-9.

Lowe, J., F. van den Ent, and L.A. Amos. 2004. Molecules of the bacterial cytosqueleton. Annu Rev Biophys Biomol Struct 33:177-98.

Lu, C., M. Reedy, and H.P. Erickson. 2000. Straight and curved conformations of FtsZ are regulated by GTP hydrolysis. J Bacteriol 182:164-70.

M Doi, M.W., F Ishino, S Tomioka, M Ito, Y Sakagami, A Suzuki, and M Matsuhashi 1988 Determinations of the DNA sequence of the mreB gene and of the gene products of the mre region that function in formation of the rod shape of Escherichia coli cells. . J. Bacteriol. 170:4619-4624.

Markiewicz, Z., J.K. Broome-Smith, U. Schwarz, and B.G. Spratt. 1982. Spherical E. coli due to elevated levels of D-alanine carboxypeptidase. Nature 297:702-4.

Martinez-Antonio, A., S.C. Janga, and D. Thieffry. 2008. Functional organisation of Escherichia coli transcriptional regulatory network. J Mol Biol 381:238-47.

Matias, V.R., A. Al-Amoudi, J. Dubochet, and T.J. Beveridge. 2003. Cryo-transmission electron microscopy of frozen-hydrated sections of Escherichia coli and Pseudomonas aeruginosa. J Bacteriol 185:6112-8.

Matin, A. 1991. The molecular basis of carbon-starvation-induced general resistance in Escherichia coli. Mol Microbiol 5:3-10.

McCoy, A.J., and A.T. Maurelli. 2006. Building the invisible wall: updating the chlamydial peptidoglycan anomaly. Trends Microbiol 14:70-7.

Meberg, B.M., A.L. Paulson, R. Priyadarshini, and K.D. Young. 2004. Endopeptidase penicillin-binding proteins 4 and 7 play auxiliary roles in determining uniform morphology of Escherichia coli. J Bacteriol 186:8326-36.

Mirelman, D., Y. Yashouv-Gan, and U. Schwarz. 1977. Regulation of murein biosynthesis and septum formation in filamentous cells of Escherichia coli PAT 84. J Bacteriol 129:1593-600.

Mirelman, D., Y. Yashouv-Gan, Y. Nuchamovitz, S. Rozenhak, and E.Z. Ron. 1978. Murein biosynthesis during a synchromous cell cycle of Escherichia coli B. J Bacteriol 134:458-61.

Moller-Jensen, J., and J. Lowe. 2005. Increasing complexity of the bacterial cytosqueleton. Curr Opin Cell Biol 17:75-81.

Moller-Jensen, J., R.B. Jensen, J. Lowe, and K. Gerdes. 2002. Prokaryotic DNA segregation by an actin-like filament. Embo J 21:3119-27.

Moller-Jensen, J., J. Borch, M. Dam, R.B. Jensen, P. Roepstorff, and K. Gerdes. 2003. Bacterial mitosis: ParM of plasmid R1 moves plasmid DNA by an actin-like insertional polymerization mechanism. Mol Cell 12:1477-87.

Mottl, H., and W. Keck. 1991. Purification of penicillin-binding protein 4 of Escherichia coli as a soluble protein by dye-affinity chromatography. Eur J Biochem 200:767-73.

Mottl, H., P. Nieland, G. de Kort, J.J. Wierenga, and W. Keck. 1992. Deletion of an additional domain located between SXXK and SXN active-site fingerprints in penicillin-binding protein 4 from Escherichia coli. J Bacteriol 174:3261-9.

Nicholas, R.A., S. Krings, J. Tomberg, G. Nicola, and C. Davies. 2003. Crystal structure of wild-type penicillin-binding protein 5 from Escherichia coli: implications for deacylation of the acyl-enzyme complex. J Biol Chem 278:52826-33.

Nikaido, H., E.Y. Rosenberg, and J. Foulds. 1983. Porin channels in Escherichia coli: studies with beta-lactams in intact cells. J Bacteriol 153:232-40.

Nystrom, T. 1999. Starvation, cessation of growth and bacterial aging. Curr Opin Microbiol 2:214-9.

Nystrom, T. 2002. Translational fidelity, protein oxidation, and senescence: lessons from bacteria. Ageing Res Rev 1:693-703.

Nystrom, T. 2003a. Conditional senescence in bacteria: death of the immortals. Mol Microbiol 48:17-23.

Nystrom, T. 2003b. The free-radical hypothesis of aging goes prokaryotic. Cell Mol Life Sci 60:1333-41.

Nystrom, T. 2003c. Nonculturable bacteria: programmed survival forms or cells at death's door? Bioessays 25:204-11.

Osborn, M.J., and L. Rothfield. 2007. Cell shape determination in Escherichia coli. Curr Opin Microbiol 10:606-10.

Phadtare, S., and M. Inouye. 2001. Role of CspC and CspE in regulation of expression of RpoS and UspA, the stress response proteins in Escherichia coli. J Bacteriol 183:1205-14.

Pichoff, S., and J. Lutkenhaus. 2007. Identification of a region of FtsA required for interaction with FtsZ. Mol Microbiol 64:1129-38.

Piette, A., C. Fraipont, T. Den Blaauwen, M.E. Aarsman, S. Pastoret, and M. Nguyen-Disteche. 2004. Structural determinants required to target penicillin-binding protein 3 to the septum of Escherichia coli. J Bacteriol 186:6110-7.

Pisabarro, A.G., M.A. de Pedro, and D. Vazquez. 1985. Structural modifications in the peptidoglycan of Escherichia coli associated with changes in the state of growth of the culture. J Bacteriol 161:238-42.

Pla, J., M. Sanchez, P. Palacios, M. Vicente, and M. Aldea. 1991. Preferential cytoplasmic location of FtsZ, a protein essential for Escherichia coli septation. Mol Microbiol 5:1681-6.

Priyadarshini, R., M.A. de Pedro, and K.D. Young. 2007. Role of peptidoglycan amidases in the development and morphology of the division septum in Escherichia coli. J Bacteriol 189:5334-47.

Raskin, D.M., and P.A. de Boer. 1999. Rapid pole-to-pole oscillation of a protein required for directing division to the middle of Escherichia coli. Proc Natl Acad Sci U S A 96:4971-6.

Reeve, C.A., A.T. Bockman, and A. Matin. 1984a. Role of protein degradation in the survival of carbon-starved Escherichia coli and Salmonella typhimurium. J Bacteriol 157:758-63.

Reeve, C.A., P.S. Amy, and A. Matin. 1984b. Role of protein synthesis in the survival of carbon-starved Escherichia coli K-12. J Bacteriol 160:1041-6.

Rice, K.C., E.E. Mann, J.L. Endres, E.C. Weiss, J.E. Cassat, M.S. Smeltzer, and K.W. Bayles. 2007. The cidA murein hydrolase regulator contributes to DNA release and biofilm development in Staphylococcus aureus. Proc Natl Acad Sci U S A 104:8113-8.

Rothfield, L. 2003. New insights into the developmental history of the bacterial cell division site. J Bacteriol 185:1125-7.

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 32:789-98.

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 157:31-8.

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 45:1729-40.

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 60:177-88.

Sauvage, E., F. Kerff, M. Terrak, J.A. Ayala, and P. Charlier. 2008. The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis. FEMS Microbiol Rev 32:234-58.

Scheffers, D.J., C. Robichon, G.J. Haan, T. den Blaauwen, G. Koningstein, E. van Bloois, J. Beckwith, and J. Luirink. 2007. Contribution of the FtsQ transmembrane segment to localization to the cell division site. J Bacteriol 189:7273-80.

Schiffer, G., and J.V. Holtje. 1999. Cloning and characterization of PBP 1C, a third member of the multimodular class A penicillin-binding proteins of Escherichia coli. J Biol Chem 274:32031-9.

Schmidt, K.L., N.D. Peterson, R.J. Kustusch, M.C. Wissel, B. Graham, G.J. Phillips, and D.S. Weiss. 2004. A predicted ABC transporter, FtsEX, is needed for cell division in Escherichia coli. J Bacteriol 186:785-93.

Schwarz, U., and W. Leutgeb. 1971. Morphogenetic aspects of murein structure and biosynthesis. J Bacteriol 106:588-95.

Soufo, H.J., and P.L. Graumann. 2003. Actin-like proteins MreB and Mbl from Bacillus subtilis are required for bipolar positioning of replication origins. Curr Biol 13:1916-20.

Spratt, B.G. 1975. Distinct penicillin binding proteins involved in the division, elongation, and shape of Escherichia coli K12. Proc Natl Acad Sci U S A 72:2999-3003.

Spratt, B.G. 1977. Properties of the penicillin-binding proteins of Escherichia coli K12. Eur J Biochem 72:341-52.

Stephens, C. 2005. Senescence: even bacteria get old. Curr Biol 15:R308-10.

Stewart, E.J., R. Madden, G. Paul, and F. Taddei. 2005. Aging and death in an organism that reproduces by morphologically symmetric division. PLoS Biol 3:e45.

Stricker, J., P. Maddox, E.D. Salmon, and H.P. Erickson. 2002. Rapid assembly dynamics of the Escherichia coli FtsZ-ring demonstrated by fluorescence recovery after photobleaching. Proc Natl Acad Sci U S A 99:3171-5.

Tatusov, R.L., D.A. Natale, I.V. Garkavtsev, T.A. Tatusova, U.T. Shankavaram, B.S. Rao, B. Kiryutin, M.Y. Galperin, N.D. Fedorova, and E.V. Koonin. 2001. The COG database: new developments in phylogenetic classification of proteins from complete genomes. Nucleic Acids Res 29:22-8.

Templin, M.F.a.H., J.V. 2004. Murein tetrapeptide LD-carboxypeptidases. In *Handbook of Proteolytic Enzymes* (2nd edn) (Barret, A.J. *et al.*, eds) 2118-2120.

Testerman, T.L., A. Vazquez-Torres, Y. Xu, J. Jones-Carson, S.J. Libby, and F.C. Fang. 2002. The alternative sigma factor sigmaE controls antioxidant defences required for Salmonella virulence and stationary-phase survival. Mol Microbiol 43:771-82.

Thibessard, A., A. Fernandez, B. Gintz, N. Leblond-Bourget, and B. Decaris. 2002. Effects of rodA and pbp2b disruption on cell morphology and oxidative stress response of Streptococcus thermophilus CNRZ368. J Bacteriol 184:2821-6.

Todd, J.A., E.J. Bone, and D.J. Ellar. 1985. The sporulation-specific penicillin-binding protein 5a from Bacillus subtilis is a DD-carboxypeptidase in vitro. Biochem J 230:825-8.

Unoson, C., and E.G. Wagner. 2008. A small SOS-induced toxin is targeted against the inner membrane in Escherichia coli. Mol Microbiol 70:258-70.

van den Bogaart, G., N. Hermans, V. Krasnikov, and B. Poolman. 2007. Protein mobility and diffusive barriers in Escherichia coli: consequences of osmotic stress. Mol Microbiol 64:858-71.

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

van den Ent, F., M. Leaver, F. Bendezu, J. Errington, P. de Boer, and J. Lowe. 2006. Dimeric structure of the cell shape protein MreC and its functional implications. Mol Microbiol 62:1631-42.

van den Ent, F., T.M. Vinkenvleugel, A. Ind, P. West, D. Veprintsev, N. Nanninga, T. den Blaauwen, and J. Lowe. 2008. Structural and mutational analysis of the cell division protein FtsQ. Mol Microbiol 68:110-23.

van der Linden, M.P., L. de Haan, M.A. Hoyer, and W. Keck. 1992. Possible role of Escherichia coli penicillin-binding protein 6 in stabilization of stationary-phase peptidoglycan. J Bacteriol 174:7572-8.

Vats, P., and L. Rothfield. 2007. Duplication and segregation of the actin (MreB) cytosqueleton during the prokaryotic cell cycle. Proc Natl Acad Sci U S A 104:17795-800.

Vieira, H.L., P. Freire, and C.M. Arraiano. 2004. Effect of Escherichia coli morphogene bolA on biofilms. Appl Environ Microbiol 70:5682-4.

Vlamis-Gardikas, A., A. Potamitou, R. Zarivach, A. Hochman, and A. Holmgren. 2002. Characterization of Escherichia coli null mutants for glutaredoxin 2. J Biol Chem 277:10861-8.

Vollmer, W., D. Blanot, and M.A. de Pedro. 2008. Peptidoglycan structure and architecture. FEMS Microbiol Rev 32:149-67.

Wachi, M., K. Osaka, T. Kohama, K. Sasaki, I. Ohtsu, N. Iwai, A. Takada, and K. Nagai. 2006. Transcriptional analysis of the Escherichia coli mreBCD genes responsible for morphogenesis and chromosome segregation. Biosci Biotechnol Biochem 70:2712-9.

Wang, S., S.J. Arends, D.S. Weiss, and E.B. Newman. 2005. A deficiency in S-adenosylmethionine synthetase interrupts assembly of the septal ring in Escherichia coli K-12. Mol Microbiol 58:791-9.

Weichart, D., N. Querfurth, M. Dreger, and R. Hengge-Aronis. 2003. Global role for ClpP-containing proteases in stationary-phase adaptation of Escherichia coli. J Bacteriol 185:115-25.

Weidel, W., H. Frank, and H.H. Martin. 1960. The rigid layer of the cell wall of Escherichia coli strain B. J Gen Microbiol 22:158-66.

Whitchurch, C.B., T. Tolker-Nielsen, P.C. Ragas, and J.S. Mattick. 2002. Extracellular DNA required for bacterial biofilm formation. Science 295:1487.

Wientjes, F.B., and N. Nanninga. 1991. On the role of the high molecular weight penicillin-binding proteins in the cell cycle of Escherichia coli. Res Microbiol 142:333-44.

Yasuda, K., H. Adachi, Y. Fujiwara, and N. Ishii. 1999. Protein carbonyl accumulation in aging dauer formation-defective (daf) mutants of Caenorhabditis elegans. J Gerontol A Biol Sci Med Sci 54:B47-51; discussion B52-3.

Yoshida, H., Y. Maki, H. Kato, H. Fujisawa, K. Izutsu, C. Wada, and A. Wada. 2002. The ribosome modulation factor (RMF) binding site on the 100S ribosome of Escherichia coli. J Biochem 132:983-9.

Zapun, A., T. Vernet, and M.G. Pinho. 2008. The different shapes of cocci. FEMS Microbiol Rev 32:345-60.

Chapter 2

BolA affects cell growth, binds to the promoters of PBP5 & 6 and regulates their expression

INDEX

BolA affects cell growth, binds to the promoters of Penicillin-Binding Proteins 5 and 6 and regulates their expression

Inês Batista Guinote[1], Rute Gonçalves Matos[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

* Correspondence to: Cecília M. Arraiano

Phone: +351 214469547

Fax: +351 214469549

E-mail: cecilia@itqb.unl.pt

Running title: BolA affects growth and regulates PBP5 and PBP6

ABSTRACT

The gene *bolA* was discovered in the 80's, but unraveling its function in the cell has proven to be a complex task. The BolA protein has pleiotropic effects over cell physiology, altering growth and morphology, inducing biofilm formation, and regulating the balance of several membrane proteins. Recently BolA was shown to be a transcription factor by repressing the expression of the *mreB* gene. The present report shows that BolA is a transcriptional regulator of the *dacA* and *dacC* genes, thus regulating both DD-carboxypeptidases PBP5 and PBP6 and thereby demonstrating the versatility of BolA as a cellular regulator. In this work, we also demonstrate that reduction of cell growth and survival can be connected to the overexpression of *bolA* gene in different *E. coli* backgrounds, particularly in exponential growth phase. The most interesting finding is that overproduction of BolA affects bacterial growth differently depending on whether the cells were inoculated directly from a plate culture or from an overnight batch culture. This strengthens the idea that BolA can be engaged in the coordination of genes that adapt the cell physiology in order to enhance cell adaptation and survival under stress conditions.

Keywords: BolA, DD-carboxypeptidase, transcriptional regulator, PBP

INTRODUCTION

The BolA-like proteins are widely conserved from prokaryotes to eukaryotes. They seem to be involved in cell proliferation or cell-cycle regulation, although their molecular function is still a matter of debate. The 13.5 kDa *E. coli* BolA protein is encoded in the 10 min region of the genetic map, and is responsible for inducing spherical morphology in rod shaped bacteria in stationary phase, possibly in a FtsZ-dependent manner (Aldea et al., 1988; Lange and Hengge-Aronis, 1991). The BolA protein contains a helix-turn-helix motif, that includes a putative DNA-binding domain, through which it can eventually interact with nucleic acids and regulate the expression of different genes (Aldea et al., 1989).

The *bolA* gene is regulated by two promoters: a weak and constitutive promoter *bolA2p* and a main promoter *bolA1p,* regulated by growth phase and/or growth rate. The expression from this "gearbox promoter" is driven by σ^S sigma factor and shows an activity inversely dependent on growth rate (Aldea et al., 1990; Aldea et al., 1988; Aldea et al., 1989). *bolA* was initially considered a stationary phase gene but later it was shown that *bolA* can also be induced in exponential phase in response to several stresses (Santos et al., 1999). Ribonuclease III can act as a positive modulator of *bolA* (Freire et al., 2006a). BolA is suggested to be implicated in the tolerance to different environmental pressures since it is expressed under stress conditions, and leads to the reduction of the surface area of cells. Moreover, *bolA* was shown to be involved in the formation of biofilms, modulation of OmpF/OmpC balance and control of the cell cycle (Freire et al., 2006b; Kim et al., 2002; Santos et al., 1999; Vieira et al., 2004). BolA may act as an inducer of cell wall biosynthetic enzymes, enhancing the expression of the mRNAs from the hydrolytic DD-carboxypeptidases Penicillin-Binding Proteins PBP5 and PBP6 and the β–lactamase AmpC (Aldea et al., 1989; Freire et al., 2009; Santos et al., 2002). In fact, PBP5 over-production, like *bolA* overexpression, has been reported to produce spherical cells (Markiewicz et al., 1982). The deletion of

PBP5 produced an accumulation of muropeptides with pentapeptide side-chains and a reduction on the thickness of the peptidoglycan layer (Santos et al., 2002). Additionally, PBP6 can change its protein levels, and it increases four times in stationary phase (Buchanan and Sowell, 1982).

Recently, BolA has been demonstrated to specifically interact with the *mreB* promoter and repress *mreB* transcription, leading to a reduction in protein levels and abnormal MreB polymerization (Freire et al., 2009). MreB protein, a structural homolog of actin, was revealed to be essential for bacterial cell elongation and rod shape (Jones et al., 2001; van den Ent et al., 2001). Moreover, the absence of both PBP5 and PBP6 influences the cellular concentration of MreB in stationary phase. This data supports the existence of a concerted regulation between the peptidoglycan polymerization machinery and the morphology maintenance systems.

In this work we have established that the high levels of BolA can be detrimental for cell morphology and viability, especially if present in the early phases of growth, where this protein levels are usually negligible. Even though too much BolA seems to be harmful, its homologues are evolutionary conserved, with the remarkable exception of Gram-positive bacteria, which includes several species that can sporulate when adverse conditions occur. It thus seems tempting to speculate that this gene and the respective protein have been maintained along the evolution to favour adaptation of cells to adverse conditions.

We also wanted to understand the role of BolA in the regulation, of the PBP5 and PBP6 hydrolytic murein proteins. These two proteins are homologues but they behave slightly differently within cells (Nelson and Young, 2001), as evaluated by the phenotypic differences in the single deletion mutants studied here. Part of the BolA function in the cell requires PBP5 or PBP6 since a double deletion mutant showed different growth and morphologies in response to the increase in BolA levels (Santos et al., 2002). When overexpressing BolA the levels of PBP5 and

PBP6 transcripts are increased. Here we have shown by Surface Plasmon Resonance that BolA directly interacts with the operator region of both *dacA* (PBP5) and *dacC* (PBP6) promoters, thereby indicating a possible wider impact of BolA as a transcriptional regulator.

METHODS

Bacterial strains, plasmids and genetic manipulations

The strains used in this study are described in Table 1. When necessary, strains were transformed with plasmid pMAK580 (Aldea et al., 1988) containing *bolA* under regulation of its own promoters. Transformations were carried out as previously described (Sambrook, 1989).

Media, growth conditions and viabilities

Luria broth (LB) and Luria agar (LA) were prepared as described previously (Miller, 1972). When required, the media were supplemented with 0.4 mM thymine, 50 mg/ml chloramphenicol, and 50 mg/ml kanamycin (all from Sigma). Optical densities were measured in an Amersham Biosciences Ultrospec®500/1100*pro* spectrophotometer at 620nm, using 10 mm light path couvettes. 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 growth curves. Batch cultures were launched in one of two ways: 1- directly from LA plate where colonies were grown 16-18 hours at 37 ºC (the plates could be stored for at least one week at 4 ºC and the behavior was reproducible); 2- from an overnight (16 h) liquid culture grown at 37 ºC and 100 r.p.m. (the *inoculi* were diluted to an optical density of 0.08 measured at 620 nm (OD_{620})). Cultures were grown aerobically at 37 ºC and 120 r.p.m. For evaluation of viability, the samples were processed in LB serial dilutions, and 100 μl 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 1000/100µl.

Table 1. Strains used in this study

Strains	Description	Reference or source	Observations
MG1693	thyA715	(Bachmann and Low, 1980)	background strain
CMA10	MG1693 + *bolA*$^+$	(Santos et al., 1999)	overexpressing *bolA* (after its own promoters) from pMAK580
ED3184	his supF	kindly provided by Noreen Murray	background strain for deletion mutants
JBS980	F– his supF recA Δ*dacA*::Kan^r	(Spratt, 1980)	*pbp5* deletion mutant based on ED3184 strain
JBS1001	F– his supF recA ΔdacC	(Broome-Smith and Spratt, 1982)	*pbp6* deletion mutant based on ED3184 strain
JBS983	F–his supF recA Δ*dacC* Δ*dacA*::Kan^r	(Broome-Smith, 1985)	*pbp5* and *pbp6* double deletion mutant based on ED3184 strain
CMA15	JBS980 + *bolA*$^+$	(Santos et al., 2002)	JBS980 overexpressing *bolA* from pMAK580
CMA16	JBS1001 + *bolA*$^+$	(Santos et al., 2002)	JBS1001 overexpressing *bolA* from pMAK580
CMA17	JBS983 + *bolA*$^+$	(Santos et al., 2002)	JBS983 overexpressing *bolA* from pMAK580
CMA 18	ED3184 + *bolA*$^+$	(this study)	ED3184 overexpressing *bolA* from pMAK580
CMA50	BL21 (DE3) + pPFA02	(Freire et al., 2009)	Novagen strain with plasmid overexpressing $(His)_6$–BolA

Microscope preparations

To observe the effect of BolA on cell 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, according to the growth curve. 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 n^{r}.1 cover glass. Images were obtained using a DMRA2 microscope (Leica) under Nomarski optics coupled to a CCD camera, with Metamorph software.

Overexpression and purification of BolA protein

The plasmid used for expression of BolA was a pET28a derived pPFA02 (Freire et al., 2009) transformed into a Novagen *E. coli* BL21 (DE3) strain (Table 1). Cells were incubated at 37 ºC in 250 ml LB medium supplemented with 100 µg/ml ampicillin to an OD_{620} of 0.5. Induction was performed with 0.5 mM IPTG during 60 min. Bacterial cells were pelleted by centrifugation at 6500 rpm for 10 min and stored at -20°C.

Purification of BolA was performed by histidine affinity chromatography using HiTrap Chelating HP columns (GE Healthcare) and AKTA fast protein liquid chromatography system (GE Healthcare). Cells were ressuspended in buffer A with 20mM Sodium Phosphate pH7.4 and 50mM NaCl and lysed using a French Press at 9,000 psi, in the presence of 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The crude extracts were treated with Benzonase (Sigma) during 30 minutes and clarified by a 15 min centrifugation at 9500 g. The clarified extracts were then added to a HiTrap Chelating Sepharose 1 ml column equilibrated in buffer A. Protein elution was achieved by a continuous imidazol gradient (until 100 mM) in the same buffer. The fractions containing purified protein were pooled together and buffer was exchanged to pure buffer A (without imidazol)

using Amicon®Ultra Centrifugal Filters 10 kDa (Millipore™). 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. The dacA (PBP5) and *dacC* (PBP6) promoters were amplified by PCR using pbp5Fw 5'-GGGGTACCGCAACGTTTGCAAACCGAAG-3', pbp5Rev 5'-CCATCGATCTGAACTACGACATCCGTG-3', pbp6Fw 5'-GGGGTACCCATACTCACCCCTTTTCC-3', and pbp6Rev 5'-CCATCGATCCACCCGAGTATCCATTC-3' primers, respectively. As a positive control, the promoter sequence of the *mreBCD* operon was used and as a negative control we tested *bolA* open reading frame (ORF) DNA encoding fragment as previously described (Freire et al., 2009). We also used, as negative controls, the ORF from *mreB*, *PBP5*, *PBP6* and *RNase II*. These regions were amplified by PCR using RTmreB 5'-ACTTGTCCATTGACCTGGGTACTG-3', RTmreB2 5'-GCCGCCGTGCATGTCGATCATTTC-3', codingpbp5Fw 5'-CCGCTCGTATCATGAAGCGCC-3', coding pbp5Rev 5'-CCGAAGAAGTTACCTTCCGGG-3', coding pbp6Fw 5'-CTCCTTCGTGGTCTTGC-3', codingpbp6Rev 5'- GATTAAGAGAACCAGCTGCCG-3', rnb_477 5'-GGCGATCGTTCTTTCTATGCAGAA-3' and Asp210Asn_Rev 5'-TAGCGAAGAGGGCGTTATCCATATCTTCTG-3' (Barbas et al., 2008). The assays were run at 25 ºC in 20 mM Sodium Phosphate pH 7.4, 1 mM dithiothreitol, and 500 mM NaCl buffer. The amplified DNA fragments were

injected over the flow cells for 2.5 minutes at a minimum of 5 different concentrations between 6.4 $x10^{-5}$ and 5 nM using a flow rate of 20 μl/min. All experiments included triple injections of each sequence concentration to determine the reproducibility of the signal. Bound protein was removed with a 60 sec wash with 50 mM NaOH. Equilibrium constants were determined using the BIA Evaluation 3.0 software package, according to the fitting model 1:1 Langmuir Binding, and χ^2 was the statistics used to measure the fitness of the model to the data.

RESULTS AND DISCUSSION

BolA overexpression impairs cell growth rate

Bacterial growth rates after inoculation depend on the growth stage at time zero. If the culture is growth arrested, cell replication will be partially delayed as metabolism has to be restarted; if, on the contrary, the culture is in exponential phase, the multiplication rate is maximal, and so there will be no lag phase. Regarding BolA expression, however, this situation is more prominent. In this case, the cells do not just have to resume or maintain growth, but it seems that they have to adapt to the levels of this protein in the cell.

In optimal growth conditions, BolA protein increases its levels when cells are entering into stationary phase (Aldea et al., 1989). Therefore, if growth is initiated after an overnight suspension culture with BolA highly expressed, BolA will be present in high concentration in the cells diluted into new media. We wanted to study the role of differential BolA levels on starting cultures. Therefore we have monitored the growth on two different backgrounds (MG1693 and ED3184), using cultures started after an overnight liquid growth (liq_), or cultures directly inoculated from the plate (pl_). Comparisons were made for the same conditions using the strains transformed with pMAK580 *bolA* overexpressing plasmid (respectively CMA10 and CMA18) (Fig.1).

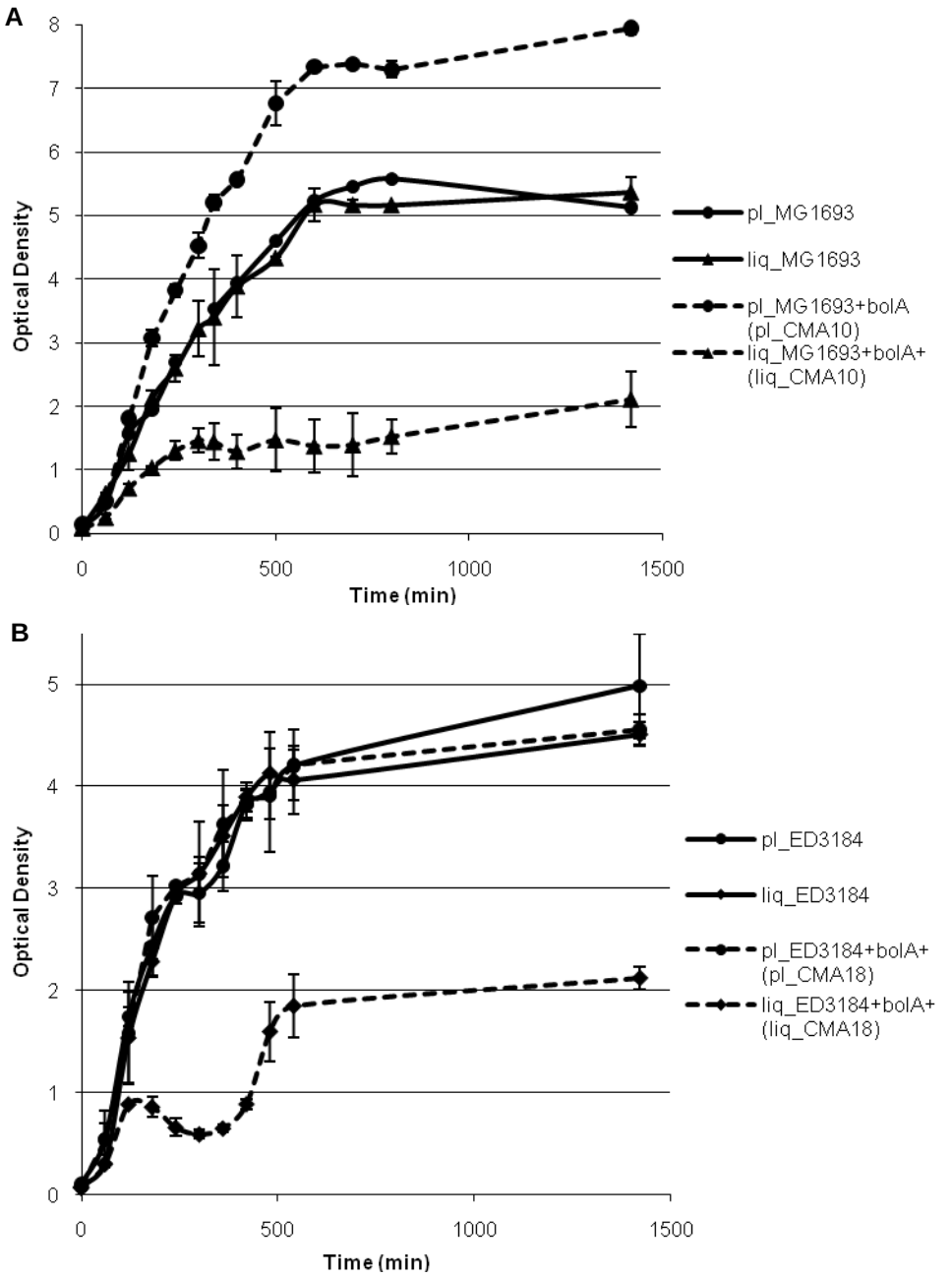


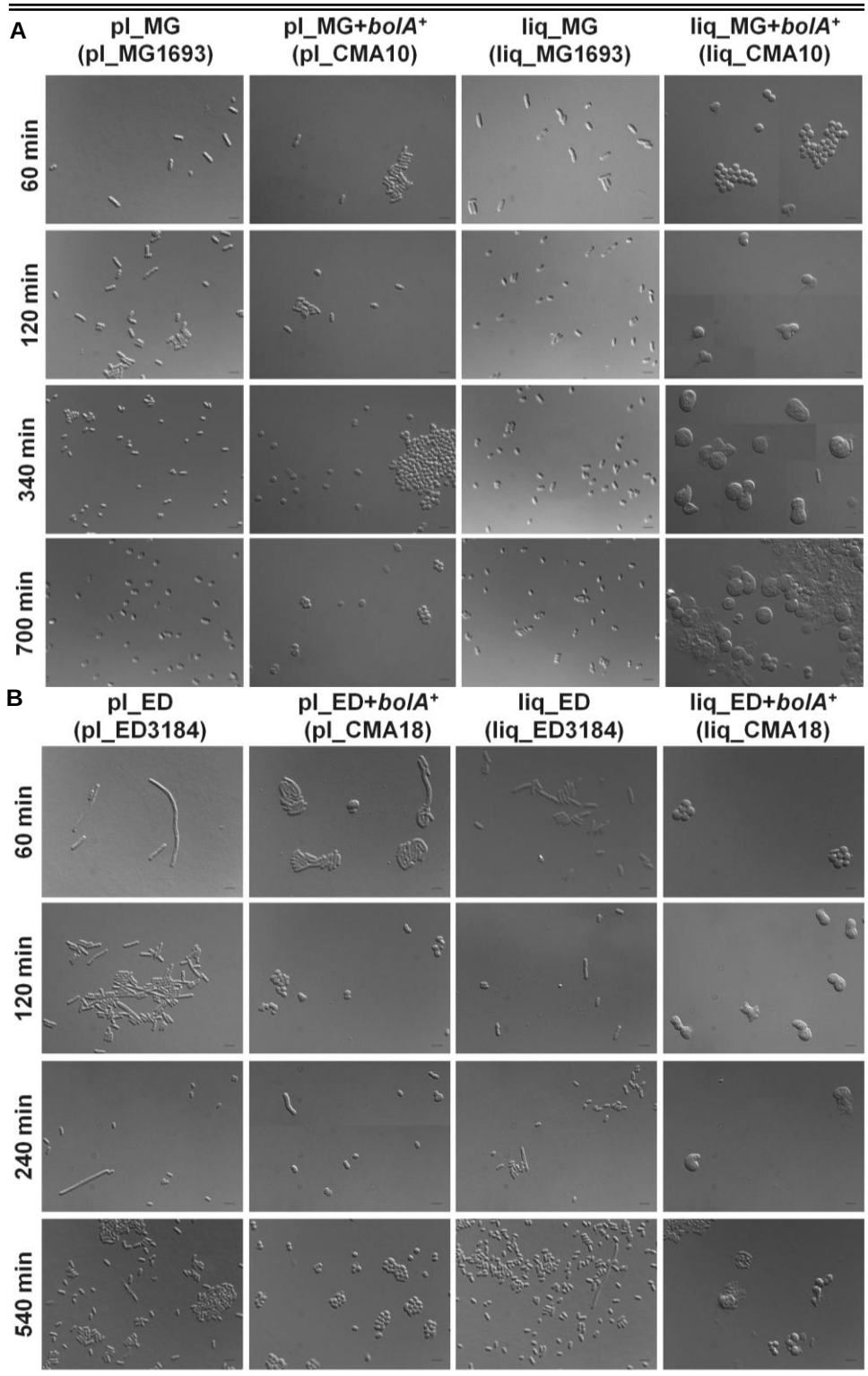
Figure 1. Optical density measurements at 620 nm for determination of growth curves in LB media supplemented according to the strains, at 120 r.p.m., 37 ºC – average and standard deviations from a minimum of three independent repetitions are presented; prefix with starting OD: (pl_) for strains directly grown from plate and (liq_) after an overnight liquid growth. **A**. MG1693 and MG1693+*bolA*$^{+}$ (CMA10) strains; **B**. ED3184 and ED3184+*bolA*$^{+}$ (CMA18) strains.

In the wild type strains the growth rate is not dependent on the starting culture (see pl_MG1693 *vs.*liq_MG1693, and pl_ED3184 *vs.* liq_ED3184, Fig. 1). Nevertheless, in the presence of additional copies of *bolA* due to the presence of pMAK580, a *bolA* overexpressing plasmid, serious changes in growth are observed depending on the *inoculum* (see pl_CMA10 (MG1693+*bolA*$^+$) *versus* liq_CMA10 (MG1693+*bolA*$^+$) and pl_CMA18 (ED3184+*bolA*$^+$) *versus* liq_CMA18 (ED3184+*bolA*$^+$)) (Fig.1). For strains bearing the plasmid (*bolA*$^+$) growth is strongly impaired for cultures started from batch culture, when compared to the cultures directly inoculated from the plate. We have compared the levels of *bolA* mRNA levels in both starters and the results showed that the levels of *bolA* mRNA are about 2.3 times higher when the *inoculum* comes from an overnight liquid growth. In this case, cultures can only support growth until half of the maximum OD value reached when they are directly grown from plate (liq_CMA10 and liq_CMA18 *vs.* pl_CMA10 and pl_CMA18) (Fig.1). This behavior is not reported for the strains without pMAK580 and occurs similarly in both MG1693 and ED3184 *E. coli* backgrounds (Fig.1).

BolA effect on growth rate is correlated with alterations in bacterial morphology

The changes in growth behavior were evaluated by microscopy analysis (Fig.2). Similarly to what has been observed in the growth curves, cell morphology does not vary much according to the growth state of the *inoculum* for the wild type strains: in MG1693 background no differences can be distinguished; and in the ED3184 strain it is possible to see a mixed filament/rods population with a propensity for rods after an overnight liquid growth (Fig.2).

Figure 2. (In the next page) Representative Differential Interference Contrast microscopy photographs overlaid in Photoshop to increase amount of data presented. DIC micrographs were obtained using a DMRA microscope (Leica) at time points 60, 120, 340, and 700 or 540 min of the growth curves. **A** MG1693 and CMA10 (MG1693+*bolA*$^+$) strains; **B**. ED3184 and CMA18 (ED3184+*bolA*$^+$) strains. The black bar represents 5 μm.


A
pl_MG
(pl_MG1693)
pl_MG+bolA+
(pl_CMA10)
liq_MG
(liq_MG1693)
liq_MG+bolA+
(liq_CMA10)
60 min
120 min
340 min
700 min
B
pl_ED
(pl_ED3184)
pl_ED+bolA+
(pl_CMA18)
liq_ED
(liq_ED3184)
liq_ED+bolA+
(liq_CMA18)
60 min
120 min
240 min
540 min

The strains carrying pMAK580 plasmid alter their morphologies from rods to spheres as advanced exponential phase is reached, when they start growth from an agar plate (pl_CMA10 and pl_CMA18) (Fig.2). However, when they are started from a suspension culture (liq_CMA10 and liq_CMA18), cells present a spherical shape even in the beginning of logarithmic phase. In this case, as long as cell division proceeds, the spherical morphologies evolve towards larger spheres that eventually bulge, or even burst (Fig.2). This may be the reason for these strains to have a lower growth rate when compared to the others.

BolA affects the PBP5 and PBP6 DD-carboxypeptidases

After establishing how bacterial growth rates and patterns depend on the origin of the inoculi, which revealed particularly important for strains where BolA is more expressed, the effects of this protein over the *E. coli* DD-carboxypeptidases Penicillin-Binding Proteins PBP5 and PBP6 were analyzed. For that purpose, strains derived from ED3184 wild type were used, namely single and double deletants for *dacA* (PBP5) and *dacC* (PBP6) genes, and those transformed with pMAK580 (see Table 1). Similarly to what had been observed for the background strains, the growth curves for the deletant mutants, with and without pMAK580, were completely superimposed when they were cultured from plated colonies (data not shown). Further analysis was performed in the conditions where strains presented pronounced phenotypic effects derived from BolA increased levels, therefore starting after overnight batch cultures.

Initially, growth curves were performed for all the strains derived from ED3184, with and without pMAK580 plasmid for overexpression of BolA. The results show that the PBP deletants JBS980 ($PBP5^-$), JBS1001 ($PBP6^-$), and JBS983 ($PBP5^-$ $PBP6^-$), follow a similar growth curve to their respective wild type strain ED3184 (Fig. 3A). However, upon BolA overexpression, all strains except the double deletant have their growth strongly impaired, mainly in early log phase, where optical density values remain constant or even decrease (see Fig. 3A).

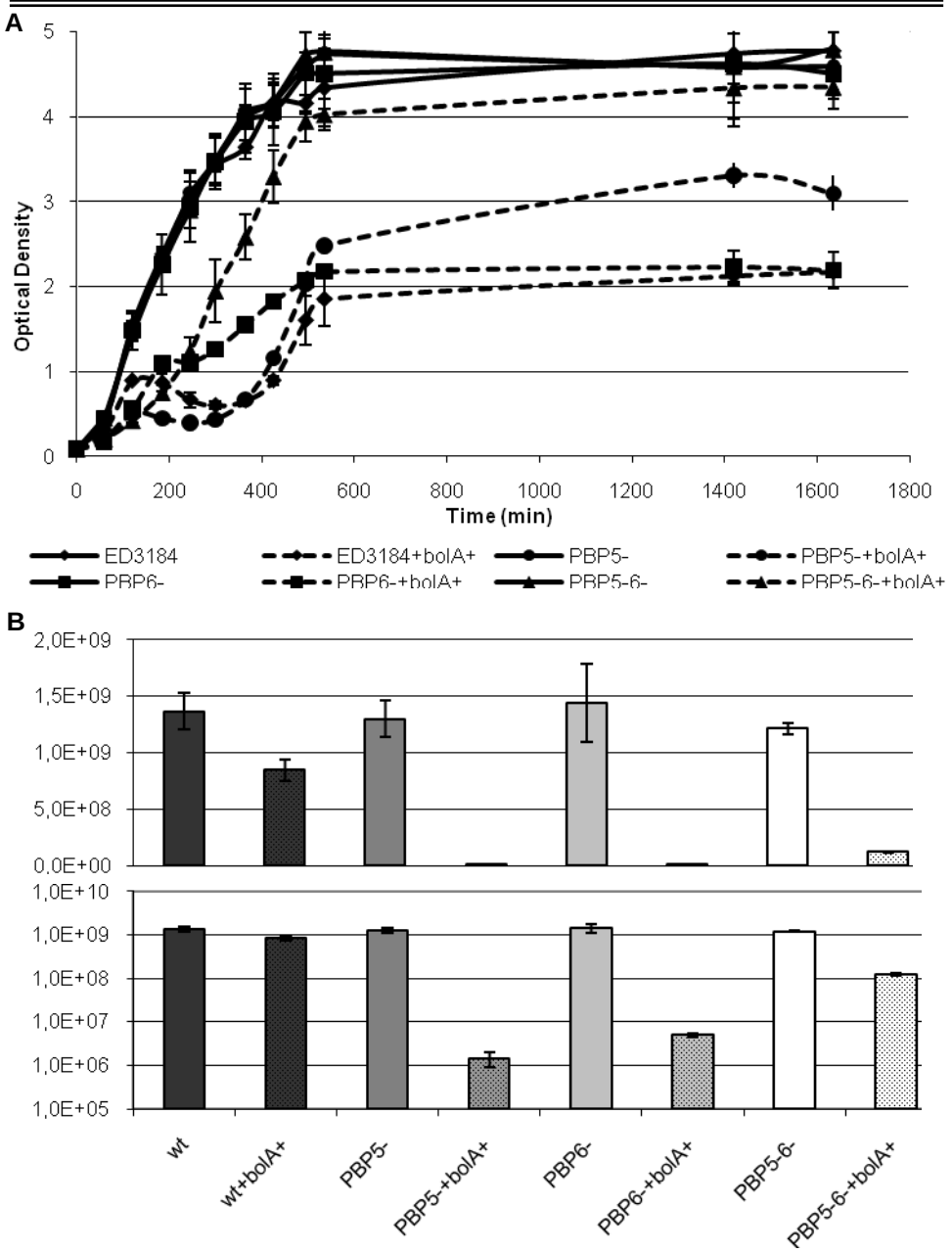


Figure 3. Growth and viability analysis for evaluation of the role of BolA on PBP mutant strains– average and standard deviations from a minimum of three independent repetitions are presented. **A**. Optical density measurements at 620nm for determination of growth curves in LB media supplemented according to the requirement of the respective strains, at 120 r.p.m., 37ºC, after an overnight liquid growth. **B**. Viability analysis at the 240 min, in decimal and logarithmic scale, according to materials and methods description.

BolA is naturally expressed in the transition from late exponential to stationary phase (Aldea et al., 1989), concomitantly with a multitude of metabolic and morphological changes in the cells (Aldea et al., 1988; Freire et al., 2009; Freire et al., 2006b; Santos et al., 2002). After a certain threshold BolA might be toxic for cells. In that sense, most cells with increased BolA levels (in strains transformed with pMAK580, particularly after overnight growth) tend to have difficulties in recovering. The cultures present an adaptation period that can be considered as an "extreme" form of lag phase. In strain liq_ED3184+bolA (liq_CMA18) while cells adapt to fresh media and restart a fast duplication stage (corresponding to exponential phase of bacterial development) a significant increase in the cells sizes occurs (see fig. 2B, liq_CMA18, times 60 to 120 minutes) increasing the OD unrelated to the changes in cell number. Those cells that significantly increase in size (the majority of the initial population inoculated) seem to become committed to a "lysis" and at about 240 minutes of growth we observe cells lysing or "bursting" (see fig. 2B, liq_CMA18, 240 minutes). The transition between 120 and 240 minutes of the growth curve should correspond to the "explosion" of the majority of high dimensioned cells. At that time, a reduced number of smaller rod shaped unsynchronized cells, already present at the *inoculum*, substitute the initial bacterial population already dividing at a fast rate, and that accounts for the subsequent increase in OD. All strains overexpressing BolA, with the exception of the double deletion mutant, present longer adaptation time to reach exponential phase. In fact, the largest difference between growth curves with and without pMAK580 is not in the rate of multiplication, after the strains are already adapted to the new growth media (Fig. 3). The rate is about the same for all, except the PBP6 single deletion strain overexpressing BolA. The latter multiplies about 2.7 times slower than all others. Differences in curves are mostly due to the time that the strains require to "adapt" BolA levels to new media conditions. In that sense, there is virtually no lag phase in cultures where pMAK580 is absent,

and while the transformed background strain CMA18 takes the longest period to adjust (7 times more), the double mutant, along with the single PBP6 mutant overexpressing BolA adapts faster (4 times faster than in the absence of the plasmid). It is possible that a partial substitution effect between PBPs might be happening, since the effects on growth due to BolA are apparently prevented in the simultaneous absence of both DD-carboxypeptidases. The data additionally suggests that PBP5 is a preferential target for BolA action. The strains where this protein is present are more affected by BolA, not only in growth rate but also in the maximum OD reached by cultures: OD_{620nm}= 2 for the PBP6 single mutant - where PBP5 is present - *versus* OD_{620nm}= 3 for the PBP5 single deletant CMA15 and 4.5 for the double mutant CMA17, where both PBPs - and BolA targets - are absent. The double mutant CMA17, apart from an increased lag time, appears to grow quite similarly to the strains without the BolA overexpressing plasmid. A possible explanation could be that this lag period may be independent from the effects of BolA over the PBPs but instead related to regulation of *mreB* by BolA. In laboratory regular growth conditions, the MreB cytoskeleton protein should be continuously expressed until later stages of the growth curve, when its levels are reduced. Nevertheless, this protein is strongly inhibited by BolA, presenting reduced concentrations in cells whenever it is being expressed (Freire et al., 2009). In this way, when cells should be ready to divide, their size is artificially reduced thus avoiding or delaying division. As a result, the fast growth phenotype and true exponential phase are concealed until BolA levels are reduced/washed out from cells, at about 240 minutes of growth, demonstrating a simple response of the division rate to the amount of MreB (Freire et al., 2009), directly dependent on the BolA levels (see Fig. 3A). Convergent conclusions can be inferred from the examination of viabilities throughout growth, with the exception of the wild type strain transformed with pMAK580 (CMA18) (Fig. 3B and 4). CMA18 growth curve seems to be strongly impaired, but it is only slightly affected in actual

viability counts (Fig. 3B). At 240 minutes of growth viable colonies for strains transformed with pMAK580 increase about one order of magnitude from the PBP5$^-$ to the PBP6$^-$ strain, and the same happens between the PBP6 single mutant and the double mutant (that shows the minimal phenotype related to BolA, when comparing all the deletants). The PBP5 deletant with enhanced BolA expression reaches increased viable counts later on, after adaptation has occurred, giving the idea that this protein might not be naturally expressed at early stationary phase. This can be clearly observed in the representative viability analysis of all strains along time (Fig. 4). The double mutant CMA17 is the one in which viability is less influenced by the increased BolA levels, while the viabilities for single PBP6 mutant are the most affected, again suggesting that in the presence of PBP5 the cells are more sensitive to BolA levels. The detrimental effects of excess of BolA protein levels are particularly observed in fast cellular replication stages, coherently with the fact that this protein is naturally expressed in reduced growth rate conditions, such as in stationary phase or upon stresses (Aldea et al., 1989; Santos et al., 1999). These results further strengthen the idea that BolA may act as a regulator for both DD-carboxypeptidases. They also reinforce the observation that this regulation might be important for the phenotypes observed upon stationary phase or stress induction.

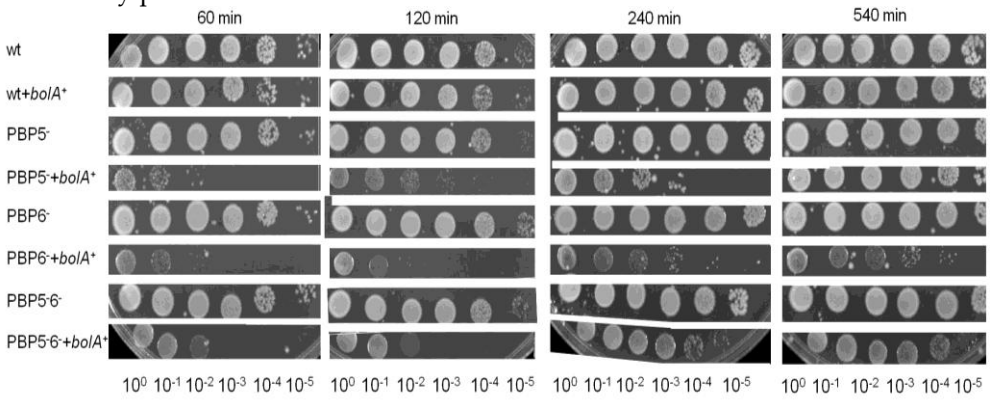


Figure 4. Representative viability analysis for the time points 60, 120, 240, and 540 min, presented as 10 μl spots of serial dilutions (10^0 to 10^{-5}) from the cultures in LB media.

It seems tempting to speculate that BolA has a preference for PBP5 as target, since when this gene is deleted, the presence of pMAK580 has a reduced impact on growth impairment and cell counts remain higher.

Morphological evaluation of these strains by optical microscopy further substantiated that the double deletion strain with pMAK580 is less sensitive to the BolA overproduction (Fig. 5). For CMA17 strain we can see that rods and even filaments are always present, and that even when some spheres appear they

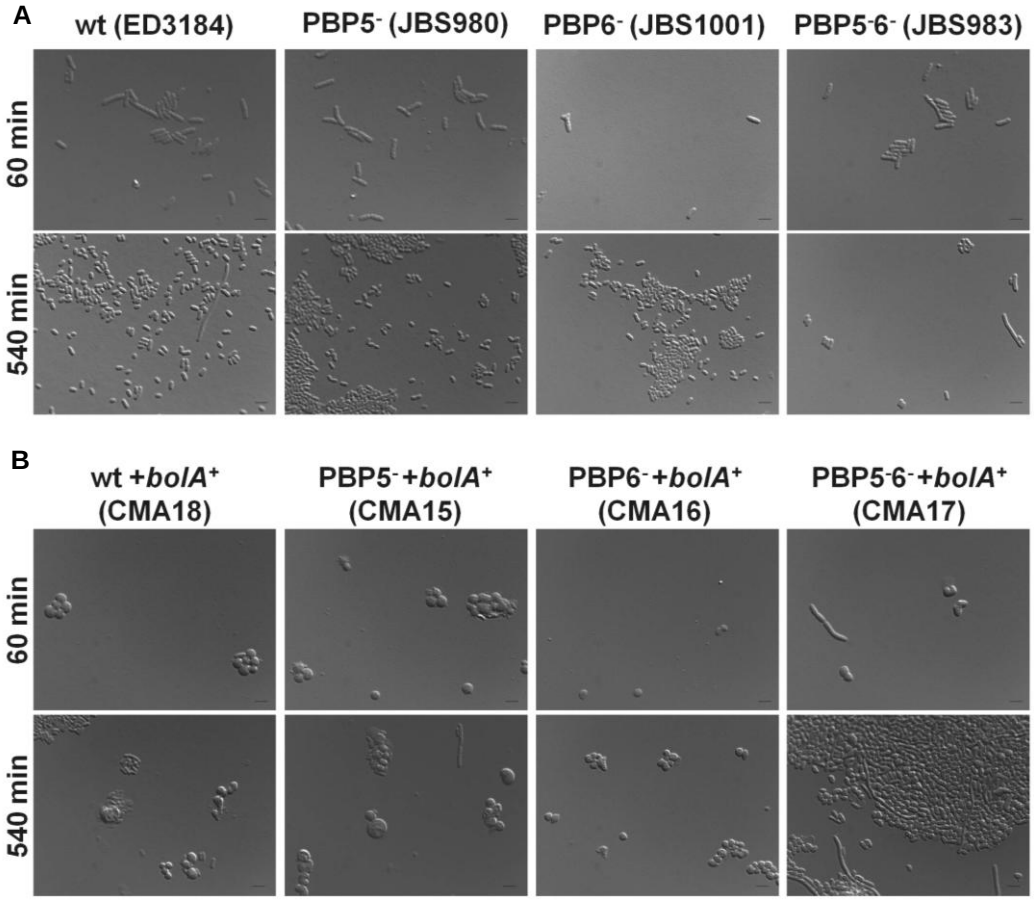


Figure 5. Representative Differential Interference Contrast microscopy photographs. obtained using a DMRA microscope (Leica) for early exponential and early stationary phase of the all strains derived from ED3184 background - **A** - and the same strains transformed with *bolA*$^+$ - **B**. The black bar represents 5 μm.

are not the dominant phenotype; however, in the other strains with the pMAK580 plasmid (CMA15, CMA16 and CMA18) the cells are essentially spherically shaped, changing in size and membrane integrity, as it can be visualized through cells bulging and/or bursting (Fig. 5). Interestingly, when PBP5 is absent, the spheres seem to become larger than for the other single mutant, showing again that PBP5 and PBP6 are not equivalent targets for BolA.

BolA interacts in vitro with the promoter regions of dacA and dacC, acting as a positive transcriptional regulator for PBP5 and PBP6

Finally, we wanted to verify if the effect of BolA over the PBP5 and PBP6 proteins (Aldea et al., 1989) and mRNA levels (Santos et al., 2002) is direct. It was previously determined that BolA directly interacts with *mreB* promoters *in vitro* (Freire et al., 2009). In that system, BolA acts repressing *mreB* transcription and strongly reduces the *mreB* mRNA and protein levels. BolA upregulation of *dacA* and *dacC* mRNA levels has already been established (Santos et al., 2002). By Surface Plasmon Resonance, we measured the affinity of BolA protein to PBP5 and PBP6 promoters. As negative controls, we used DNA fragments amplified from the coding region of both PBPs (PBP5 ORF and PBP6 ORF) and also from a different gene, *rnb*, which encodes for the ribonuclease II (RNase II), an enzyme that degrades RNA molecules (RNase II ORF). As a positive control, we used the promoter region of *mreB*, previously demonstrated to have affinity for BolA protein (Freire et al., 2009). The results obtained are presented in Table 2. The dissociation constant value obtained for the *mreB* promoter in these new experiments corresponds to the one previously published (6.9 nM) (Freire et al., 2009). The obtained data shows that BolA protein has slightly more affinity for *dacA* and *dacC* than for the *mreB* promoter (1.8nM and 5.3 nM versus 6.9 nM, respectively) (Table 2). In all cases, the affinity of BolA is significantly higher for the promoter regions than for the coding regions (120 nM e 102 nM, respectively). These results confirm that BolA behaves as a general transcriptional regulator.

Furthermore, it can act either as a repressor of *mreB*, or as an activator of gene expression for production of PBP5 and PBP6. It is also possible to detect that BolA has more affinity for PBP5 promoter when compared to the others (1.8 nM versus 6.9 nM and 5.3nM) (Table 2). According to the van´t Hoff equation: $\Delta G^0 = RT\ln K_D$, where R and T are the universal gas constant and absolute temperature, we determined the Gibbs free energy difference, ΔG^0. The values obtained are also reported in Table 2. The determination of the ΔG^0 informs us about the likelihood of complex formation. If ΔG^0 is negative, then we are in the presence of a spontaneous reaction. All the BolA protein-DNA interaction tests present a negative $\Delta G°$, which means that all these reactions are spontaneous and can occur *in vivo*. However, for the PBP5 promoter interaction, an even lower value is obtained when compared to the others (Table 2), meaning that the binding of BolA to the PBP5 promoter is much more probable. The binding to the coding regions PBP5 ORF, PBP6 ORF and RNase II ORF, on the other hand, is not so favourable since they present the highest ΔG^0 values (Table 2). If we analyze the other equilibrium constants, we can see that the BolA protein interacts with the three promoters in a different way. The association rate constant (k_a) gives us information about the kinetics of association, i.e., how fast the complex is formed, while the dissociation rate constant (k_d) relates to the dissociation kinetics or the velocity of complex dissociation. For the MreB promoter, the association is fast (k_a of 1.1×10^5 /M.s) and the dissociation is slow (k_d of 7.43×10^{-4} /s). For the PBP5 promoter, the association is not as fast as for the MreB promoter (k_a of 7.5×10^4 /M.s) but the dissociation is also slower (k_d of 1.3×10^{-4} /M.s) (Table 2) showing that the PBP5 promoter DNA-BolA complex is more stable than the MreB promoter DNA-BolA. However, for the PBP6 promoter, the results are quite different: the association of BolA to the promoter is really slow and the same is observed for the dissociation, which is much slower than the ones observed for the MreB or PBP5 promoters (Table 2). This behavior may indicate that the

complex BolA-PBP6 promoter once bound can be even more stable when compared to the other complexes. For the coding regions tested, we can observe that the association is very slow, which reflects the poor affinity of BolA to these regions (Table 2).

Overall SPR experimental results confirm that BolA acts as a transcriptional regulator of *dacA*, *dacC*, and *mreB*. They particularly contribute to understand why the single deletion mutants for the PBP5 and PBP6 proteins present such physiological differences in response to BolA accumulation, as reported in this work. An increase of the *dacA* (PBP5) and *dacC* (PBP6) mRNA levels had previously been observed when BolA was overexpressed (Santos et al., 2002), therefore we can hypothesize that BolA is acting as an activator of *dacA* and *dacC*.

Table 2. BolA binding affinity for different promoter (Prom) and coding regions (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^0 values were determined according to the van't Hoff equation: $\Delta G^0 = RT\ln K_D$, where R and T are the universal gas constant and absolute temperature.

	k_a (1/Ms)	k_d (1/s)	K_D (nM)	χ^2	ΔG^0 (KJ/mol)
MreB Prom	1.1×10^5	7.4×10^{-4}	6.9	1.3	-46.6
PBP5 Prom	7.5×10^4	1.3×10^{-4}	1.8	0.8	-50.0
PBP5 ORF	8.4×10^1	1.0×10^{-5}	120	1.1	-39.5
PBP6 Prom	1.5×10^3	8.1×10^{-6}	5.3	1.7	-47.2
PBP6 ORF	9.8×10^1	9.9×10^{-6}	102	1.1	-39.9
RNase II ORF	2.8×10^1	1.0×10^{-5}	365	1.6	-36.8

CONCLUSION

BolA is a protein whose levels strongly impact cellular growth rate, and the pattern of growth curves/ability to grow in rich LB media. The effects of overexpression of BolA result in aberrant cell morphologies, initially inducing formation of small spheres that then evolve into large spheres and aberrant morphologies. It was demonstrated that the phenotypes regarding the overexpression of *bolA* are much more prominent if the *inoculum* is taken from an overnight liquid culture, in which BolA has been considerably expressed and accumulated. It had been previously proposed that BolA might be related with hydrolytic DD-carboxypeptidases Penicillin-Binding Proteins PBP5 and PBP6. An increase of the *dacA* (PBP5) and *dacC* (PBP6) mRNA levels had previously been established (Santos et al., 2002) and a strain where *bolA* gene was deleted seemed to show a decrease in PBP5 or PBP6 protein levels (Aldea et al., 1989). In this study BolA was shown to be a more broad range transcriptional regulator directly interacting not only with the promoter region of *mreB* reducing its expression levels, but also with the promoter regions of the genes that code for the murein cross-linking enzymes PBP5 and PBP6.

This work has opened new perspectives for the impact of BolA in bacterial growth and survival. Since BolA is able to target different genes as a transcriptional regulator, having the capacity of acting either as a repressor or as an activator, it is now important to determine to which other targets it can bind and regulate. Furthermore it is also required to analyze how BolA regulation might connect with sigma factors and affect global transcriptional machinery in stress conditions. This will allow us to determine to which extent BolA can modulate the cell and facilitate adaptation to less than optimal growth conditions.

ACKNOWLEDGEMENTS

We would like to thank Noreen Murray for all her kindness and continuous efforts to find and provide us the strain ED3184. IBG and RGM were recipients of Doctoral fellowships from FCT (Fundação para a Ciência e Tecnologia - Portugal). 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.

REFERENCES

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 9:3787-94.

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 170:5169-76.

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 8:3923-31.

Bachmann, B.J., and K.B. Low. 1980. Linkage map of Escherichia coli K-12, edition 6. Microbiol Rev 44:1-56.

Barbas, A., R.G. Matos, M. Amblar, E. Lopez-Vinas, P. Gomez-Puertas, and C.M. Arraiano. 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 283:13070-6.

Broome-Smith, J.K. 1985. Construction of a mutant of Escherichia coli that has deletions of both the penicillin-binding protein 5 and 6 genes. J Gen Microbiol 131:2115-8.

Broome-Smith, J.K., and B.G. Spratt. 1982. Deletion of the penicillin-binding protein 6 gene of Escherichia coli. J Bacteriol 152:904-6.

Buchanan, C.E., and M.O. Sowell. 1982. Synthesis of penicillin-binding protein 6 by stationary-phase Escherichia coli. J Bacteriol 151:491-4.

Freire, P., R.N. Moreira, and C.M. Arraiano. 2009. BolA inhibits cell elongation and regulates MreB expression levels. J Mol Biol 385:1345-51.

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 88:341-6.

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 260:106-11.

Jones, L.J., R. Carballido-Lopez, and J. Errington. 2001. Control of cell shape in bacteria: helical, actin-like filaments in Bacillus subtilis. Cell 104:913-22.

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 14:425-30.

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 173:4474-81.

Markiewicz, Z., J.K. Broome-Smith, U. Schwarz, and B.G. Spratt. 1982. Spherical E. coli due to elevated levels of D-alanine carboxypeptidase. Nature 297:702-4.

Miller, J.H. 1972. Experiments in Molecular Genetics Cold

Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Nelson, D.E., and K.D. Young. 2001. Contributions of PBP 5 and DD-carboxypeptidase penicillin binding proteins to maintenance of cell shape in Escherichia coli. J Bacteriol 183:3055-64.

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.

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 32:789-98.

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 45:1729-40.

Spratt, B.G. 1980. Deletion of the penicillin-binding protein 5 gene of Escherichia coli. J Bacteriol 144:1190-2.

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 70:5682-4.

Chapter 3

BolA can contribute to dormancy, protecting cells against external stresses in E. coli: is bolA a new persister gene?

INDEX

BolA can contribute to dormancy, protecting cells against external stresses in *E. coli*: is *bolA* a new persister gene?

Inês Batista Guinote[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

✉ Correspondence to: Cecília M. Arraiano

Phone: +351 214469547

Fax: +351 214469549

E-mail: cecilia@itqb.unl.pt

Running title: BolA - a new "persister gene"?

Keywords: BolA, transcriptional regulator, persistence, phenotypical differentiation, stress survival, tolerance

ABSTRACT

Microorganisms have evolved to divide and maintain in harsh conditions. Several external stresses are dealt with by bacteria in a daily basis, many times through biofilm formation. The adaptation mechanisms usually imply cell surface and/or structure changes in shape, dimensions, cell wall thickness, protein content, and permeability. The *E. coli* gene *bolA* is involved in morphological transformations and is mainly expressed in stress conditions. BolA is a good candidate to evaluate adaptation mechanisms to unfavourable environments. In this report we have evaluated BolA function as a potential bacterial persister protein, declining the multiplication potential and providing tolerance against different stresses imposed, creating a multiplication potential in a long term perspective.

INTRODUCTION

Bacteria are constantly facing environmental challenges. Membrane plasticity is important to evaluate bacterial surroundings and facilitate exchanges; more rigid and less permeable boundaries, nevertheless, promote survival when coping with stress conditions. The stationary phase gene gene *bolA* (Aldea et al., 1990; Aldea et al., 1989) is growth phase-regulated and controlled by the sigma factor σ^S (*rpoS* gene) (Lange and Hengge-Aronis, 1991). Its expression is also induced in response to several stresses, in a partially σ^S-independent manner (Santos et al., 1999). *E. coli bolA* is a morphogene that induces spherical shape of cells when overexpressed (Aldea et al., 1988). This effect of *bolA* is mediated by the induction of either PBP5 and/or PBP6 D,D-carboxypeptidases (Aldea et al., 1988; Guinote et al., 2010; Santos et al., 2002) and repression of the expression of bacterial actin homologue MreB (Carballido-Lopez, 2006; Freire et al., 2009). The increased expression of *bolA* not only reduces the exposed surface but also increases the ratio volume to area, modulates cell permeability (Freire et al., 2006b) and induces the formation of biofilms (Vieira et al., 2004) the ultimate protection structure against external damaging agents. Some bacteria can survive different kinds of stresses without particular gene resistances. They have the ability to escape lethality by becoming dormant, this is stopping cellular metabolism and division. For instance, when antibiotics are added to a system they are expected to interact with the proteins they have affinity with, in a way that prevents their correct function. If there are persister cells in the system where the antibiotics are applied, the antibiotics molecular targets are practically inactive, and thus, binding will not lead to any effect. The antibiotics will decontaminate the system of the proliferating cells but not of the persisters, that latter on regain the ability to proliferate (Lewis, 2007). The existence of persisters has been acknowledged since the middle of the XXth century (Bigger, 1944). The ability to form persister cells

was furthermore shown to differ along the growth curve: it is decreased in the beginning, presents a sharp increase in the middle of the exponential phase, and reaches its maximum (about 1%) in stationary phase cells. This dependence on the bacterial physiological state was confirmed by the inability to create persisters when cultures were diluted four times only reaching exponential phase of growth (Keren et al., 2004). This dynamics seems to rely on the inherent phenotypic heterogeneity of the bacterial populations, which may be dependent on the differential accumulation levels of "persister" protein(s), elicited by different environmental factors that affect the killing process (Lewis, 2007). The expression of *E. coli bolA* has been extensively studied and is tightly controlled both at the transcriptional and post-transcriptional levels, having an expression consistent with those of persister proteins (Freire et al., 2006a; Lange and Hengge-Aronis, 1991; Santos et al., 2006). This 13.5 kDa protein is the most studied member of the BolA-like family. It contains only one defined domain named BolA/YrbA, essentially formed by a putative DNA-binding helix-turn-helix (HTH) motif (Aldea et al., 1989). In this study we assessed both physiological induction of *bolA* as it happens along the growth curve, and artificially imposed high expression of BolA events. The results led us to acknowledge that this protein can act in two complementary ways. An initial survival mechanism is induced when low to medium amounts of BolA are present in the cell. Medium levels of BolA lead to the formation of small spheres. The reduction of surface to volume ratio makes them less susceptible to be affected by the action of certain toxics. It furthermore reduces the division constraining the population increase and reducing the consumption of resources. A posterior mechanism is triggered when high levels of this protein are present in cells, leading them to progressively increase dimensions, deforme and finally burst. This lysis releases cellular components that may provide a small part of the population the scavenger molecules for toxics, the adhesion properties for biofilm development and protection, or the

nutrients lacking in the medium for stalled cells to become metabolically active. A desynchronization may account for the absence of relative loss of viability in such severe conditions enabling the "rebirth" of cells average sized, regular and rod-like shaped. Moreover we could show here the protective effect of BolA favouring bacteria survival against the antimicrobial nalidixic acid. This preliminary work opens the possibility that *bolA* might be a "new" persistence gene.

METHODS

Bacterial strains, plasmids and genetic manipulations

The strains used in this study are described in Table 1. MG1693 chromosomal DNA was used as a template for amplification of *bolA* Open Reading Frame (ORF). The product of the PCR reaction was inserted into Bluescript pSK plasmid (PROMEGA) to construct plasmid pPFA01 and transformed into strain DH5α. Plasmid pPFA01 was used to obtain the *bolA* ORF flanked with *NdeI*/*BamHI* restricted sites to be ligated in pET28a (Novagen) originating plasmid pPFA02. *E. coli* strain BL21(DE3) was transformed with pPFA02 to overexpress $(His)_6$-BolA.

Table 1. Strains used in this study.

Strains	Description	Reference or source	Observations
MG1693	thyA715	Bachmann and Low, 1980	background strain
CMA83	MG1693 + pBr325	(this study)	mother plasmid
CMA10	MG1693 + pMAK580	Santos et al., 1999	overexpressing bolA from plasmid
DH5α			commercial strain
CMA61	DH5α+pPFA01	(Freire, 2005)	intermediate plasmid with *bolA* after *NdeI* sequence
CMA62	DH5α+pPFA02	(Freire, 2005)	pET15b based plasmid with *bolA* inducible by IPTG
BL21(DE3)			Novagen strain
CMA50	BL21 (DE3) + pPFA02	(Freire et al., 2009)	BL21 with plasmid over-expressing (His)6–BolA

Transformations were carried out as previously described (Sambrook, 1989). BolA overexpression was induced with a final concentration of 0,5mM IPTG and growth was continued for 30 min, 1h and 2h to assess protein expression levels.

Construction of the (His)$_6$-BolA expressing vector pPFA02

The ORF coding for BolA protein was generated by polymerase chain reaction (PCR) amplification with *Pfu* polymerase from Fermentas, using *E. coli* genomic DNA (strain MG1693) as template. DNA template was prepared using the genomic DNA purification kit from PUREGENE™DNA Cell & Tissue Kit Purification System from Gentra Systems. Forward primer BolAp1 (5′-CGCCATATGATGATACGTGAGCGG -3′) containing a *NdeI* restriction site and reverse primer BolAp2 (5′- GCGGAATTCGACAGTTGCAAATGCG -3′) containing a *BamHI* restriction site were used in this reaction. The PCR was done in a volume of 50 μl, in the presence of 0.5 ng of template DNA, 1 ng of forward and reverse primers, and 1 unit of *Pfu* polymerase (Fermentas), during 35 cycles (1 min at 94°C, 2 min at 55°C, and 2 min at 72°C). The resulting product was cloned in a blunt-ended Bluescript pSK plasmid backbone (PROMEGA) digested by *EcoRV*, named pPFA01. That plasmid was digested with *NdeI* and *BamHI*, and after purification of the fragment containing *bolA* ORF, it was ligated to a pET-28a plasmid (Novagen) *NdeI* and *BamHI* backbone fragment, and transformed into strain BL21 (DE3). *E. coli* BL21 (DE3) competent strain (Novagen) was used for the transformation of the new expression vector construct pPFA02 containing the gene encoding for *bolA* under control of a T7 polymerase IPTG inducible promoter. The cloned gene was confirmed by oriented PCR, fragments analysis in agarose gel and sequencing by company STAB Vida, Portugal (data not shown).

Media, and growth conditions

Luria broth, and Luria agar compositions were prepared as described previously (Miller, 1972).When required, the media were supplemented with 0.4 mM thymine, 50 mg/ml chloramphenicol, and 50 mg/ml kanamycin (all from Sigma).

Optical Densities (OD) were measured at 620nm and determined according to the Lambert-Beer law's limits for the direct proportionality between OD and sample concentration (dilutions were made so that density values would be read between 0.02 and 0.6); phases of growth to be analyzed were determined according to growth curves. Batch cultures were either launched directly from previous LA plate grown colonies or from overnight growths at 37ºC and 100 r.p.m., that were diluted to an optical density of 0.08 measured at 620nm (OD_{620nm}). Cultures were grown aerobically at 37ºC and 120 r.p.m. For viability evaluation, the samples were subjected to several LB serial dilutions, and plated in the growth media added of agar. The number of colony forming units was counted and viability was determined according to the equation: Number of dividing cells per ml = cfu x $10^{dilution}$ x 1000/100µl.

Microscope preparations

To observe the effect of BolA on morphology, cells were harvested from cultures growing in LB, at 60, 210, 340, 700, and 1420 time points of the growth curve and every 30 minutes after OD_{620nm}=0.3 was reached and different conditions were imposed, to evaluate the response to stress challenge. Cells were fixated with 0.75% formaldehyde and stored at 4ºC. For the Differential Interference Contrast microscopy photographs, 20 µl of the samples were put onto slides coated with a thin 1.5% agarose film, and enclosed with n^r.1 cover glass. Images were obtained using a DMRA microscope (Leica) under phase contrast or Nomarski optics coupled to a CCD camera, with Metamorph software.

RESULTS AND DISCUSSION

BolA increased expression by pMAK580 affects cell growth rate and viabilities.

Given that the deletion of *bolA* has proven unsuccessful for the determination of its function (data not shown), similarly to what occurs for most of the genes potentially involved in persistence, eventually due to partial compensation by

other genes (Lewis, 2007), a parallel attempt was done through gain-of-function. *E. coli* MG1693 was transformed with pMAK580, a pBR325 based plasmid that encodes *bolA* under its own promoters (Aldea et al., 1988), therefore enhancing the transcript levels. Even though this is only a medium copy plasmid, there were phenotypic effects quite distinct from those of the pBR325 transformed cells. When culture was started by dilution of an overnight growth, the maximum OD reached by the CMA10 (the pMAK580 transformed strain) was about 1, while an OD of about 4 to 5 was attained in the other strains (Fig. 1A). Surprisingly, if they were to start from a glycerol stock at -80ºC, the lag time until cells were able to divide would be higher, but once it growth started high rates until an OD of 6 or 7 occured (Fig.1A). The same was seen for the cultures directly grown from glycerol stocks, although with an increased lag time that takes for cells to resume metabolism. As for the controls, MG1693 and MG1693 transformed with pBr325 strains, similar growth curves were recorded independently on the origin of the *inocula* (except for the higher lag time that cultures started from glycerol stocks took) (Fig. 1A). The viabilities were in agreement with the results of the growth curves, apart from the *bolA* overexpression strain which exhibited a strong viability reduction (Fig. 1B). In this way, BolA was demonstrated to be responsible for serious decrease in the ability of cells to reproduce, although not completely abolishing bacterial multiplication. It seems like a sort of stalling is induced in its presence, since we can always detect some amount of colony forming units throughout the growth curve. BolA could be responsible for the reduction of cell division rate to allow a minimum of cellular maintenance until better conditions come.

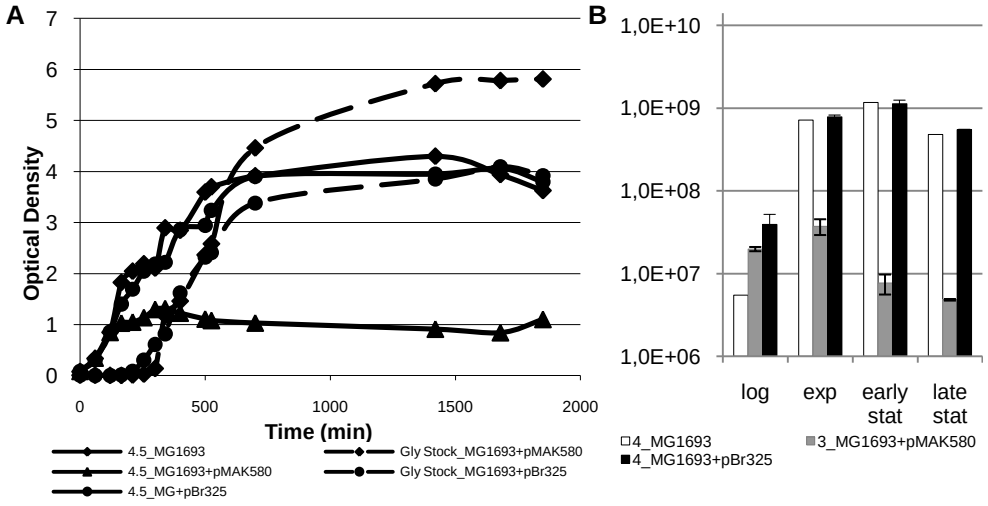


Figure 1. Growth curves and viabilities depend on *bolA* levels. A. Optical Density measurements at 620nm for determination of growth curves in properly supplemented LB media, at 120 r.p.m., 37ºC; MG1693, CMA83 (pBr325 transformed) and CMA10 (pMAK580 transformed) strains; suffix according to starting culture OD_{620nm}; B. Viability evaluated by number of colony forming units in properly supplemented LA plates for the same strains and conditions (cell counts.ml^{-1} are in logarithmic scale).

BolA levels strongly impact cell morphology and induce a phenotypic desynchronization

To check the phenotypical effects of different amounts of BolA along the growth curve, microscopy studies were performed in an experiment of serial growth after a direct *inoculum* from plate colonies into liquid media (0) from which culture new ones would be started, by dilution into new properly supplemented LB media, after reaching the ODs: 0.5, 1.5, 3, and 7. In the planning of this experiment two aspects were simultaneously addressed: the physiological state of the cultures, and the amount of BolA present in the cells when new growth was started. Not many differences could be perceived in the growth as well as in the morphologies of the MG1693 background strain when growth was started from plate (referred as OD 0), or the liquid culture at OD 1.5 or 5 (Figure 2A). On the opposite, when MG1693+pMAK580 were grown after different OD values reached, the growth patterns were different according to the conditions

(Figure 2A). When growth was started after plate, the curve presented a regular pattern but with much higher growth rate and maximum optical density.

Even though the evolution of ODs does not vary considerably for CMA10 strain started after ODs 0.5 and 1.5, the viabilities present significant differences (Figure 2B). The 0.5 culture showed less cell counts along the curve comparing to the culture started at 1.5. The latter, in turn, presented unlikely high viabilities in the first time points evaluated corresponding to the early exponential phase. We believe the improved ability to multiply in this latter culture is due to the culture being already dividing/more metabolically active and BolA levels are still not overbearing. As growth proceeds increasing levels of BolA accumulate due to the presence of the overexpression plasmid. Finally the OD3 started culture has a highly initial growth and multiplication rate but by the 120 minutes of growth the initial BolA induced boost starts to reveal its "toxicity" effects and the OD evolution relies solely on the dimensions attained by cells, not growth. At OD 7 led the maximum OD reached by cultures is about 1.5 and growth starts only about 24 hours after time 0 (Figure 1A and 2A). That moment seems to be for this strain like a time 0 for the others – cells loose the aberrant dimensions, become rod shaped again, ODs restart growing and cell count number enlarges instead of decreasing like for all other strains and conditions evaluated. It seems that, for cultures starting growth with some although amounts of accumulated BolA protein (not so much as becoming actually toxic), there is a significant percentage of cells that are maintained in culture although not dividing (Nystrom, 2003).

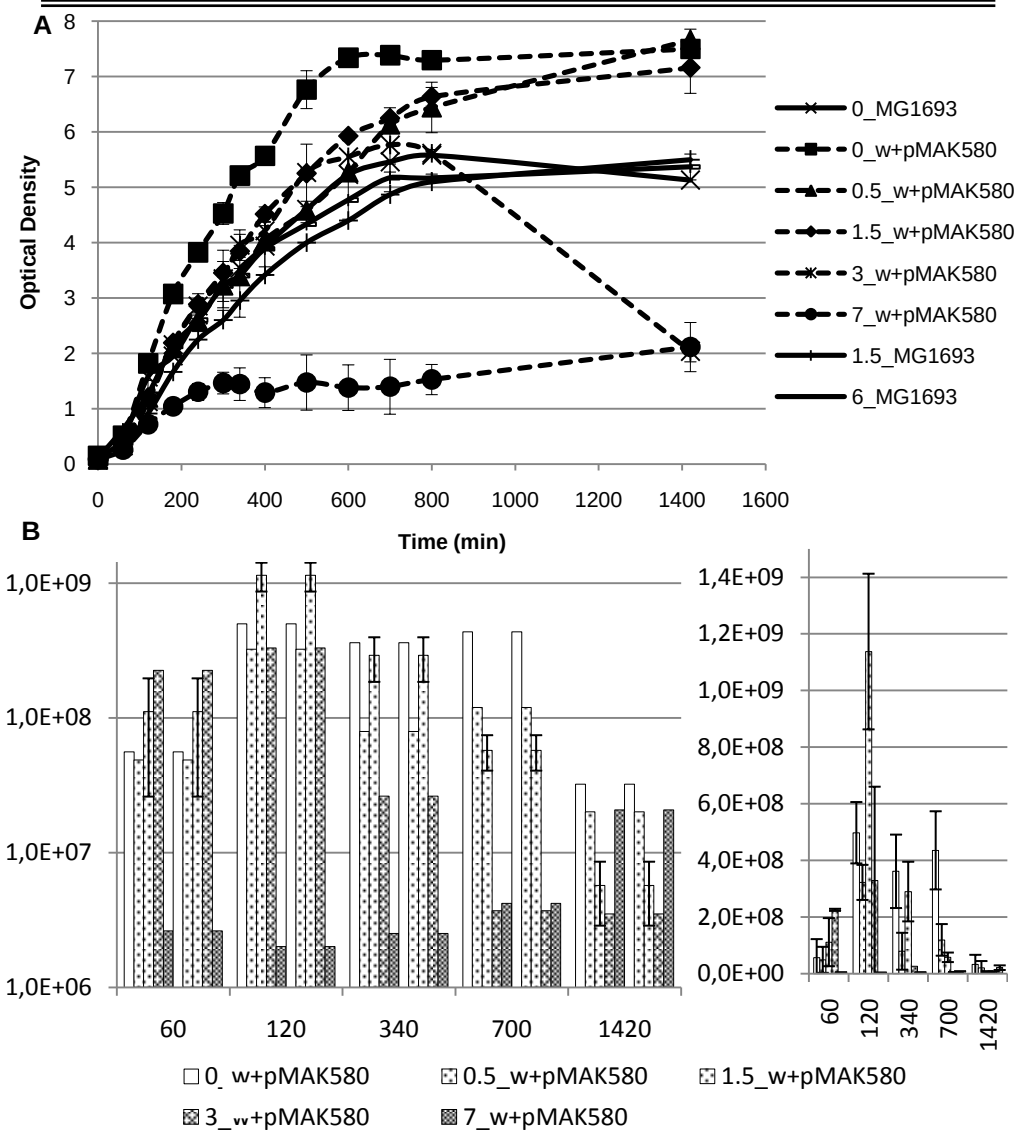


Figure 2. Serial growth curves and viabilities. A. Optical Density measurements at 620nm for determination of growth curves in LB media supplemented according to the strains, at 120 r.p.m., 37ºC; CMA10 (MG1693 transformed with pMAK580) strain followed by the starting OD: (OD 0) for strains directly grown from plate and (OD 0.5, 1.5, 3, and 7) when an optical density of 0.5, 1.5, 3, and 7 had been reached, respectively. B. Viability for the same strains and conditions evaluated by number of colony forming units in properly supplemented LA plates for the same strains and conditions.

When their growth is initiated after a more significant accumulation of BolA, it seems like it has a toxic effect that not only prevents cells from dividing, but also

induces some lyses, in a way that the OD diminishes. Since *bolA* is a morphogene, it was performed morphological evaluation to confirm if different growth patterns corresponded to differential phenotypes. Making use of this methodology we could see that the background strain does not have significant changes according to the conditions imposed (Figure 3A). The morphology of the CMA10 strain, however, strongly reflected the consequences of the different starters (Figure 3B). One could imagine the images put sequentially in the moments related to the ODs of the first culture (grown from plate). The amplification of morphological changes along time gave the rise to pear-like, lemon-like, filament-like, bulging and gigantic spheres-like morphologies (Fig. 3B). When the starter comes from OD3 or higher, bursting cells and what seems to be adhesion compounds (probably released from those lysing cells) are also detected (Figure 3B). This allows us to define a condition above which cells seem to no longer be able to redirect metabolism and cellular functions to survival; where too much external pressure or amount of internal damage leads to cell death, accounting for the cells to become gigantic, bulging and bursting.

After all of the processes described above, a surprising cellular desynchronization mechanism may be responsible for the "rebirth" or "resurrection"of small regular rods at about the same time. These rods eventually come from cells that had their metabolism stalled and regain activity due to new nutritional input or other signalling from those "programmed" to lysis.

BolA overexpression after IPTG induction reduces the rate of cell growth

In order to confirm the results described above in an independent system, a plasmid was constructed containing *bolA* under an IPTG inducible promoter (see Materials and Methods). Through addition of IPTG we could determine the effects of extra BolA in cells, independently of their physiology or development.

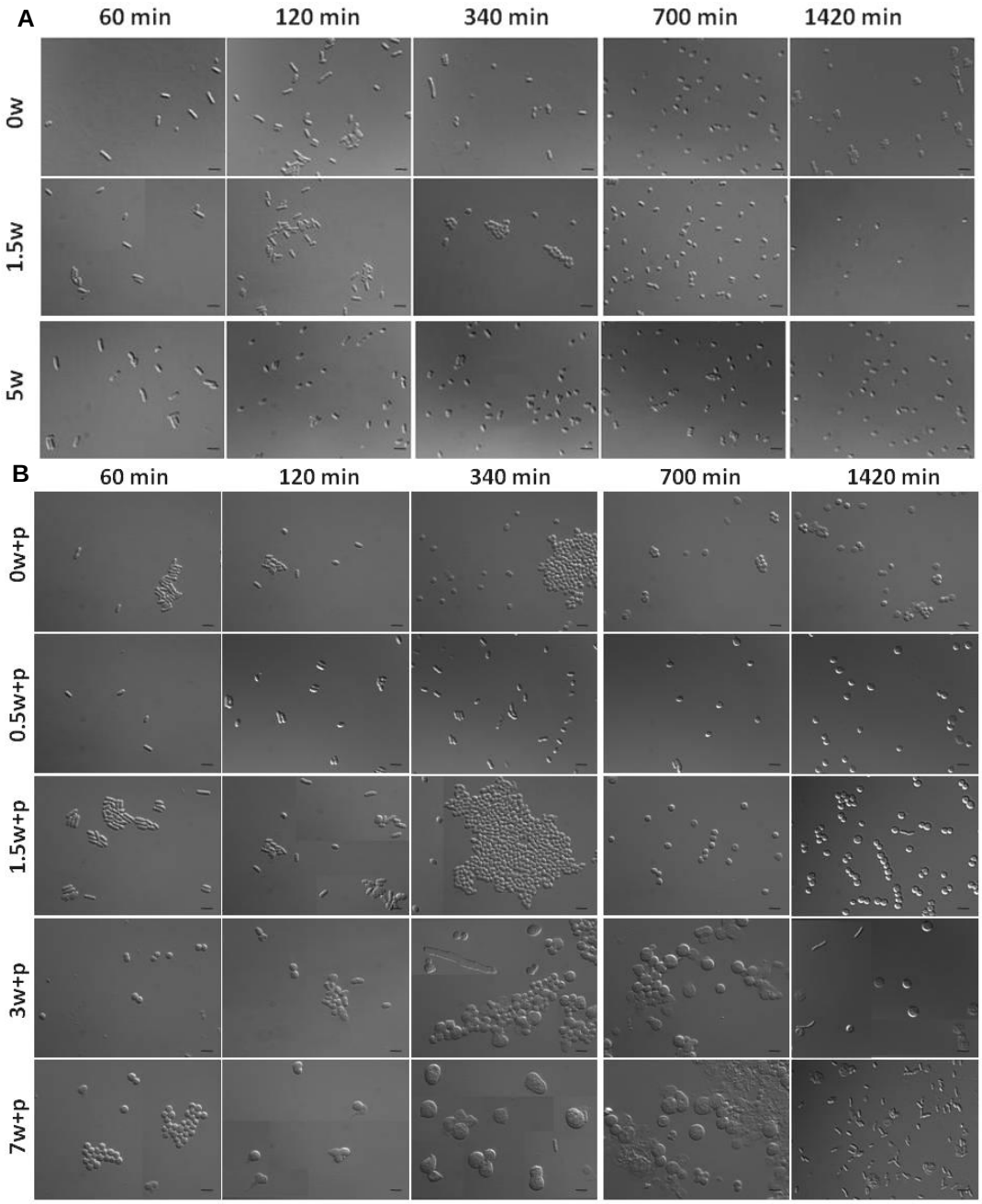


Figure 3. Differential Interference Contrast microscopy photographs, obtained using a DMRA microscope (Leica) for time points 60, 120, 340, 700, and 1420 min of the growth curves; suffix according to starting OD: (OD 0) for strains directly grown form plate and (OD 0.5, 1.5, 3, and 7) when an optical density of 0.5, 1.5, 3, and 7 had been reached. A. MG1693 strain. B. CMA10 strain.

The pPFA02 plasmid was used to analyze the influence of *bolA* overexpression perfectly growing cells, in exponential phase. *E. coli* BL21 cells were grown until early exponential phase and then IPTG was added to induce *bolA* expression (Fig. 4). About 2h after *bolA* induction, the $OD_{620\ nm}$ increased negligibly providing a growth rate of only 1.25 cell divisions per hour. Without induction of *bolA* the OD value increased around 6x, reaching a growth rate of 3.5 cell divisions per hour. The addition of glucose was intended to prevent any possible leaky transcription from the plasmid without induction. However, the growth curve results seem to show that the absence of IPTG is enough to prevent induction of *bolA* under the experimental conditions used - the system does not seem leaky. The use of IPTG by itself could not be accounted for any influence on this rate variation observed since BL21 cells control growth with and without ITPG did not show differences (Fig. 4). Therefore, the overexpression of *bolA* is sufficient to reduce significantly cell growth rate. The effects of BolA on cell morphology and membrane metabolism are probably responsible for this reduction, since they might interfere with normal cell division processes through the influence on peptidoglycan metabolism (increase of PBP5/6 expression levels) and/or the negative impact on the correct establishment of the internal cytoskeleton (downregulation of MreB protein) (Freire et al., 2009; Guinote et al., 2010). This effect can nevertheless be an important asset for bacteria in poor growth conditions or under severe stress since a slower growth rate implies a better management of cellular energetic resources and might provide the extra time necessary for bacteria to survive until conditions improve. It can furthermore prevent deleterious actions from toxics, absence of nutrients, and antimicrobials. *Escherichia coli* are essentially enterobacteria, and as such, must adapt to changes in the availability of nutrients but even more importantly to different environmental and stress conditions (pH variations, secondary metabolites, temperature, *etc.*).

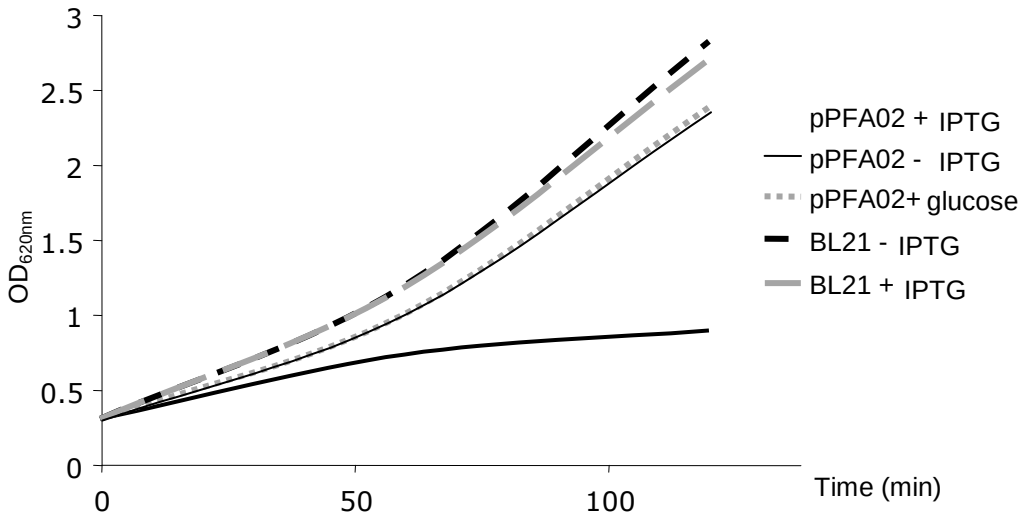


Figure 4. Analysis of the influence of *bolA* on the growth rate. The BL21 strain was used in these experiments transformed with the pPFA02 plasmid when needed (Red, orange and green curves). The influence of IPTG on general cell growth was also checked. The green curve represents the growth of BL 21+pPFA02 supplemented with glucose as a control to prevent any transcription of *bolA* due to the possible leakiness of the IPTG inducible system used. IPTG or glucose addition to the media was performed at 0.3 OD_{620}.

BolA might thus play an important role in ensuring the transition between all these different stages of growth, allowing a better adaptation to the next set of conditions.

BolA overexpression reduces filament morphology

No parallelism can be established with the physiological function of BolA, unless this recombinant protein is capable of exerting the same effect on the cellular morphology as the native BolA protein. To assess if $(His)_6$-BolA retains the morphogenetic effect of BolA, microscopy observations were performed in phase contrast (Fig. 5). $(His)_6$-BolA appears to retain the functionality of BolA since microscopic analysis clearly showed that, after 2 hours of induction, all the cells become round or olive-shaped presenting the typical phenotype of the overexpression of *bolA*. $(His)_6$-BolA is therefore active and can be used to infer the

effects of bolA overexpression as described below. BL21+pPFA02 cells contain a majority of regular rod-shaped bacteria but in this background there are also some cells forming filaments (Fig. 5). This occurs unspecifically along the growth curve, indistinctly of exponential or stationary phases of growth (data not shown). BL21 strain is depleted of the *lon* gene, a major inhibitor of the SOS response through the proteolysis of SulA protein (Mizusawa and Gottesman, 1983). This means that higher levels of SulA, a repressor of cell septation by inhibiting polymerization of FtsZ proteins, might be present in this strain upon activation of the SOS response. Since cell division becomes arrested, these cells spontaneously produce filaments due to natural impairment of DNA separation in some cells and subsequent activation of the SOS pathway.

The presence of filaments in non-induced BL21+pPFA02 cultures shows that some cells indeed present this behaviour. After BolA overexpression with IPTG in exponential phase for 120 min, the filament morphology totally disappears from the phenotype of the population and all cells become progressively rounder along the time, contrasting with the ones grown in the absence of IPTG (Fig. 5). BolA induction appears to completely repress and even reverse the formation of

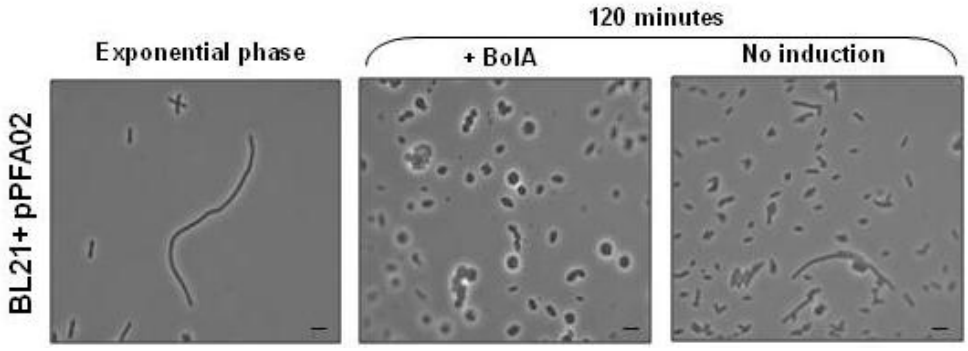


Figure 5. Phase-contrast microscopy photographs of BL21+pPFA02 strain in the presence and absence of IPTG, to induce *bolA* overexpression, 120 minutes after OD_{620nm}=0.3 was reached. The strain can presents filaments and rods. This behavior is maintained after 120 minutes of growth, in the absence of IPTG. Upon BolA expression, nevertheless, cells become spheres. This phenotype is already visible after 60 min of induction (data not shown). The dark bar represents 5 μm.

filaments since all cells present a short rounder phenotype at least 2h after induction. BolA is thus acting as a suppressor of the SOS response-derived phenotype possibly through a negative effect on cell elongation mechanisms that could be related to its repression of MreB polymerisation (Freire et al., 2009). This shows that the effect of BolA on cellular mechanisms is able to cancel other pathways. Since *bolA* is a stress response gene, highly induced in general stress conditions, this observation implies that BolA overexpression might be able to promote a reduction of cell growth and an alteration of cell morphology to spherical shape in extreme conditions, thus striving to increase cell survival.

BolA prolongs cell survival under acute stress conditions

Nalidixic acid was used to induce SOS response in the whole population of BL21 cells in the culture. Nalidixic acid is a synthetic narrow-spectrum quinolone antibacterial known to specifically inhibit synthesis of bacterial DNA, and induce cell filamentation followed by cellular death through lysis (Bauernfeind and Grummer, 1965). Since BolA may have a protective effect on cell survival according to the previously discussed results, the impact of its overexpression on cultures treated with nalidixic acid was examined. BL21+pPFA02 cultures were grown in rich LB medium until 0.3 $OD_{620\ nm}$ when nalidixic acid (Nal) was added. A control culture without Nal presents a typical growth while the addition of the antibiotic is detrimental to cell survival (Fig. 6). One hour after addition, Nal causes an arrest on cell growth followed by a fast decrease in the OD, eventually leading to the death of the population. When 60 min of treatment with Nal is followed by induction of *bolA* by IPTG, BL21 strain is able to resist to the immediate effect of the antibiotic and prolong survival (Fig. 6).

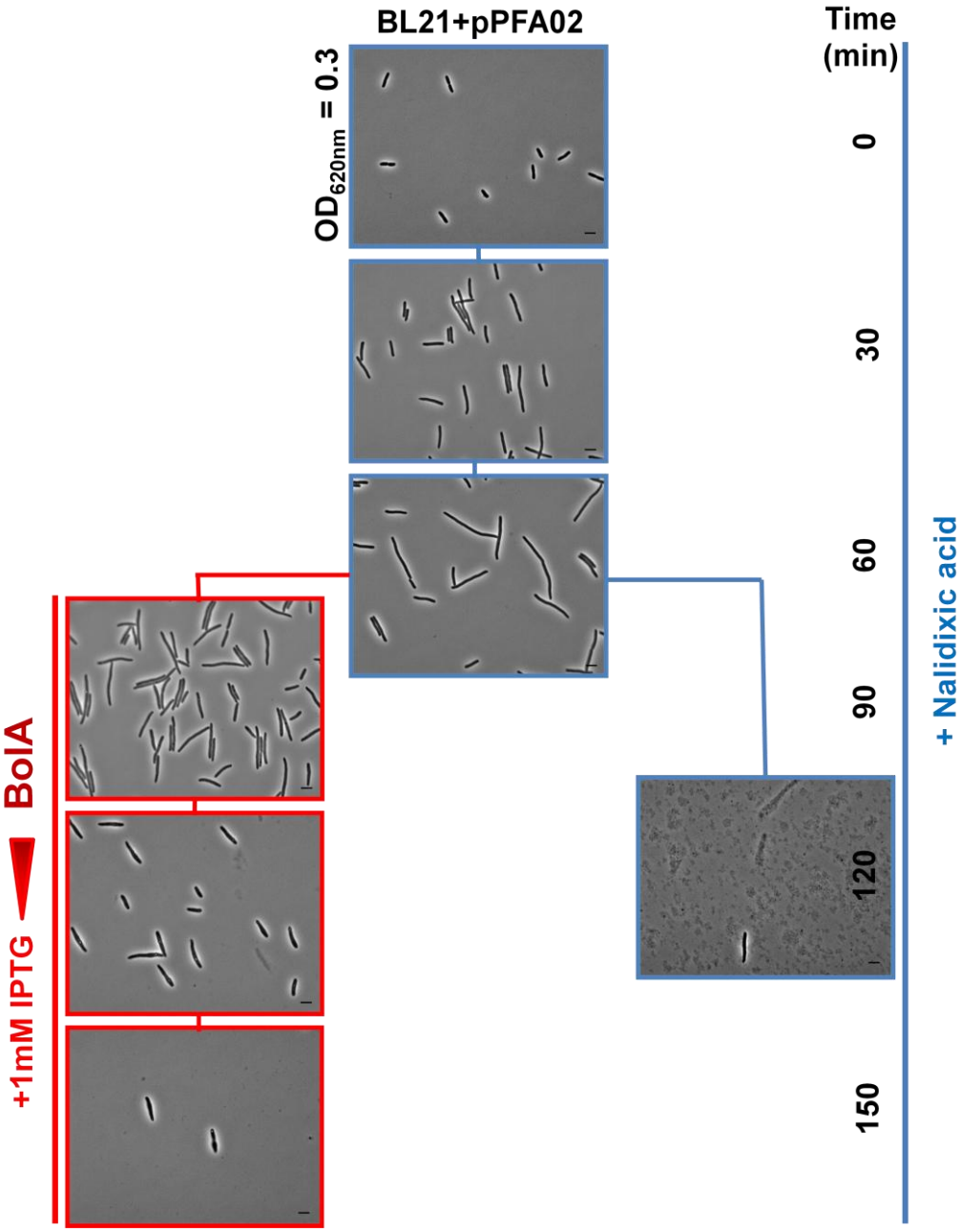


Figure 6. Phase-contrast microscopy photographs of BL21+pPFA02 cells treated with Nal at the OD_{620nm}= 0.3. The dark line represents the culture where no treatment besides Nal was added. The red lines show the morphology evolution of cell where IPTG was added 60 minutes after Nal treatment. The time is considered 0 min at each new addition.

This confirms that BolA induction (by overexpression or in response to stresses) has a potential protective effect on the cell leading to a prolonged survival of the bacteria. Furthermore, its potential to overcome and even antagonize the effects of other cellular responses already in place, as it is the case here for the SOS response, point towards a stress-response pathway centered on BolA regulation, suggesting its expression to be a cellular last resort. It has indeed been described that overproduction of BolA becomes eventually lethal (Aldea et al., 1988), something that would not be surprising if one was imaging an analogue situation to the eukaryotic programmed cell death, where the cell initially reacts to protect from toxics and avoid death, surrendering to fatality once there is too much damage accumulation. The cellular effect of *bolA* induction in the cell appears thus to be related to its concentration: a protective effect at increasing but lower concentrations, and a lethal effect when a certain threshold (higher concentration) is reached. These concentration-dependent effects of BolA might be related to its effect on the expression of different genes (Freire et al., 2009; Santos et al., 2002). They would also be of vital importance in the biofilm related pattern of cell death (Allesen-Holm et al., 2006) which requires inner cell products release and adhesion molecules (Rice et al., 2007) as well as biosorption protection compounds to be spread (Harrison et al., 2007), all of which are strongly related to the ability tolerate a multitude of stresses (Lewis, 2007).

FINAL REMARKS

BolA homologues are widely distributed among different kingdoms of life, except in Gram-positive bacteria. In this report, we have shown that overexpression of BolA is able to slow the cellular growth rate and even apparently stop division in a significant part of *E. coli* population. Our results also show that BolA induction appears to completely inhibit the spontaneous formation of filaments observed in part of the population in BL21 cultures. This effect is probably mediated by the

negative impact of BolA on cellular elongation mechanisms, namely through the direct repression of MreB expression leading to a deficient formation of the internal cytoskeleton of the cell (Freire et al., 2009). However, this reduction of cell growth rate can turn into a precious asset when in stress conditions, since cell division is delayed and more time is provided for cells to survive against metabolic toxics and reorganize the energetic pools. Cells treated with nalidixic acid become indeed more resistant to this antibiotic when BolA is overexpressed, significantly delaying the rate of cell death. BolA assumes therefore a protective role of the cell in stress conditions when its expression levels are induced. BolA is nevertheless toxic to the cell in high concentration as it was previously hypothesised (Aldea et al., 1988) and confirmed with mechanistic details in this study. These different functions depending on the concentration of BolA could indicate a more general role as gene expression regulator, possibly through the DNA binding properties predicted for the HTH domain of this protein. This has already been shown regarding the repression of *mreB* and activation of *dacA* and *dacC* genes (Freire et al., 2009; Guinote et al., 2010). BolA assumes therefore an important role in ensuring a prolonged survival of the cell in difficult growing conditions and as a mediator of general stress response. BolA relevance for cell tolerance in stress conditions becomes therefore very high, and this has particular relevance in the ecological niches of *E. coli* such as the animal or human guts. Its importance grows even further while being revealed as a new putative persistence gene (Lewis, 2007). Therefore, BolA may be involved in the mechanisms that confer microorganisms tolerance against the hosts immune system and antimicrobials toxicity, responsible for the gradual rise of chronic infections.

REFERENCES

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 9:3787-94.

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 170:5169-76.

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 8:3923-31.

Allesen-Holm, M., K.B. Barken, L. Yang, M. Klausen, J.S. Webb, S. Kjelleberg, S. Molin, M. Givskov, and T. Tolker-Nielsen. 2006. A characterization of DNA release in Pseudomonas aeruginosa cultures and biofilms. Mol Microbiol 59:1114-28.

Bauernfeind, A., and G. Grummer. 1965. Biochemical effects of nalidixic acid on Escherichia coli. Chemotherapy 10:95-102.

Bigger, J. 1944. Treatment of staphylococcal infections with penicillin by intermittent sterilization The Lancet 244 497-500.

Carballido-Lopez, R. 2006. Orchestrating bacterial cell morphogenesis. Mol Microbiol 60:815-9.

Freire, P., (ed.) 2005. Characterization of the gene *bolA* and its function in cell morphology and survival.

Freire, P., R.N. Moreira, and C.M. Arraiano. 2009. BolA inhibits cell elongation and regulates MreB expression levels. J Mol Biol 385:1345-51.

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 88:341-6.

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 260:106-11.

Guinote, I.B., R.G. Matos, P. Freire, and C.M. Arraiano. 2010. BolA affects growth and binds to the promoters of Penicillin-Binding Proteins 5 and 6 regulating their expression. Journal of Microbiology and Biotechnology:*in press*.

Harrison, J.J., H. Ceri, and R.J. Turner. 2007. Multimetal resistance and tolerance in microbial biofilms. Nat Rev Microbiol 5:928-38.

Keren, I., N. Kaldalu, A. Spoering, Y. Wang, and K. Lewis. 2004. Persister cells and tolerance to antimicrobials. FEMS Microbiol Lett 230:13-8.

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 173:4474-81.

Lewis, K. 2007. Persister cells, dormancy and infectious disease. Nat Rev Microbiol 5:48-56.

Miller, J.H. 1972. Experiments in Molecular Genetics Cold

Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Mizusawa, S., and S. Gottesman. 1983. Protein degradation in Escherichia coli: the lon gene controls the stability of sulA protein. Proc Natl Acad Sci U S A 80:358-62.

Nystrom, T. 2003. Nonculturable bacteria: programmed survival forms or cells at death's door? Bioessays 25:204-11.

Rice, K.C., E.E. Mann, J.L. Endres, E.C. Weiss, J.E. Cassat, M.S. Smeltzer, and K.W. Bayles. 2007. The cidA murein hydrolase regulator contributes to DNA release and biofilm development in Staphylococcus aureus. Proc Natl Acad Sci U S A 104:8113-8.

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.

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 32:789-98.

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 45:1729-40.

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 60:177-88.

Vieira, H.L., P. Freire, and C.M. Arraiano. 2004. Effect of Escherichia coli morphogene bolA on biofilms. Appl Environ Microbiol 70:5682-4.

Chapter 4

Characterization of the BolA homologue, IbaG: a new gene involved in acid resistance

INDEX

Characterization of the BolA homologue, IbaG: a new gene involved in acid resistance

Inês Batista Guinote[1], Ricardo Neves Moreira, 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

✉ Correspondence to: Cecília M. Arraiano

Phone: +351 214469547

Fax: +351 214469549

E-mail: cecilia@itqb.unl.pt

Running title: *ibaG*: a new gene involved in acid resistance

Keywords: *yrbA*, BolA, acid stress

Abbreviations: OM – outer membrane; ORF - open reading frame

ABSTRACT

The BolA protein homologues are widely distributed in nature. In this report, we have studied YrbA as a BolA homologue protein, and we have renamed it as *ibaG* for "induced by acid gene". *IbaG* phenotypes are nevertheless different from the *bolA* associated morphological or growth profiles. The *ibaG* and *bolA* single and double deletion mutants grew faster and had higher viabilities, in rich medium, and the *ibaG* overexpression strain exhibited no differences in morphologies, independently of the culture media. BolA seems to assume a protective role when stress conditions are imposed to cultures. In those conditions *bolA* expression levels are the highest. In this work, *ibaG* was also demonstrated to strongly increase its mRNA levels in response to acidic stress, while changing its transcript pattern. *ibaG* hydrochloric acid response may represent a new mechanism for cell resistance against acid stress.

INTRODUCTION

The BolA-like proteins are widely conserved from prokaryotes to eukaryotes. Although the molecular function is still an open field to research, those proteins seem to be involved in cell proliferation or cell-cycle regulation. The overall topology of a mouse BolA-like protein is alphabetabetaalphaalphabetaalpha, in which beta(1) and beta(2) are antiparallel, and beta(3) is parallel to beta(2). This fold is similar to the class II KH fold, except for the absence of the GXXG loop, usually well conserved. *Escherichia coli* protein sequence further exhibits a helix-turn-helix motif that may correspond to a DNA-binding domain, through which it can eventually interact and transcriptionally regulate different genes (Aldea et al., 1989). Interestingly, all the conserved residues in the BolA-like proteins are assembled on the one side of the protein (Kasai et al., 2004).

BolA, the *E. coli* 13.5 KDa morphogene protein is encoded at the 10 min region of the genetic map. It is responsible for the size reduction and spherical morphology adaptation of *E. coli*. The rod to sphere shape modulation occurs from exponentially growing to stationary phase cells, in a FtsZ (but not RodA, PBP2 or FtsQ) dependent manner (Aldea et al., 1988) (Lange and Hengge-Aronis, 1991).

The *bolA* is structurally preceded by two promoters: *bolA2p* and *bolA1p*. The upstream promoter is weak and constitutive dependent on σ^{70}. The *bolA1p* is expression driven by σ^{S} dependent gearboxes, showing an activity inversely dependent on growth rate (Aldea et al., 1990; Aldea et al., 1988; Aldea et al., 1989). In fact, *bolA* transcription has been shown to increase over 10 times (50 if values are normalized to the cell mass) and protein synthesis about 10 times in the transition to stationary phase. Besides the gearbox pattern of induction, the σ^{S} dependent promoter *bolA1p* is also induced in exponential phase in response to several stresses (heat, acid, oxidative, osmotic and glucose depletion), and stress induction seems to be partially σ^{S}–independent (Aldea et al., 1989) (Santos et al., 1999).

RNase III acts as a positive modulator of *rpoS* mRNA and σ^S levels, and therefore affects *bolA* induction (Santos et al., 1997) (Freire et al., 2006a). Also polyadenylation, which inversely correlates with bacterial growth rate (Jasiecki and Wegrzyn, 2003), reduces RssB-mediated ClpXP σ^S proteolysis, increasing the expression of the σ^S regulon genes and consequently *bolA* (Santos et al., 2006).

Escherichia coli BolA protein is already acknowledged amongst several transcriptional factors in *E. coli* (Martinez-Antonio et al., 2008). BolA was shown to repress the actin-like *E. coli* protein MreB (Freire et al., 2009) and to induce the DD-carboxypeptidases PBP5 and PBP6 (Guinote et al., 2010; Santos et al., 2002). Moreover, *bolA* overexpression induces biofilm formation, especially in stationary phase, alters the outer membrane properties, accessibility and sensitivity towards detergents and antibiotics. Those biochemical and physiological alterations may depend on BolA regulation over Inner Membrane Proteins and modulation of OmpF/OmpC balance (Aldea et al., 1989) (Vieira et al., 2004) (Freire et al., 2006b). Being strongly expressed under stress conditions and quite fastly repressed when the surroundings improve, *bolA* is suggested to be involved in promoting general resistance mechanisms. In agreement with this proposal, the *bolA* yeast homologue is a UV-inducible gene who accelerates spore germination, decreases proliferation rate, enhances cell size in vegetative growth, controls the correct septum formation and cytokinesis, confers UV resistance and is eventually responsible for the control of cell division, especially on resumption from cell cycle arrest (Kim et al., 2002). An initial characterization of a BolA homologue in *Pseudomonas fluorescens*, revealed a mild phenotype related to the sulphur metabolism. Although *bolA* has been evolutionarily predicted to be a mono-thiol glutaredoxin interacting reductase the registered phenotype for *P. fluorescens* could be solely due to the simultaneous inactivation of the two upstream operon genes rather than *bolA*.

Given the importance of BolA in several challenging environments *in silico* analysis was performed and a homologue protein (YrbA) was encountered at http://blast.ncbi.nlm.nih.gov. In fact, YrbA is described to be a BolA homologue, particularly conserved at the *bolA*/*yrbA* domain. Although gene similarity is basically absent, 23% of aminoacid overall identity, and 58% of similarity at the BolA/YrbA domain were found, and over 70% of the aminoacid residues of both proteins were aligned at http://www.pdb.org/. Moreover, similarly to BolA, YrbA was demonstrated to bear a helix-turn-helix motif, usually responsible for protein-DNA interaction, strengthening the idea that it could be accounted for the functional substitution of BolA (Fig. 1).

In this work we created a single deletion mutant for *yrbA* and a double deletion mutant for *bolA*/*yrbA* genes in *Escherichia coli*. We also produced an increased expression vector based on pBr325, where the *yrbA* gene was cloned along with its native promoter region, similarly to pMAK580 (Aldea et al., 1988).

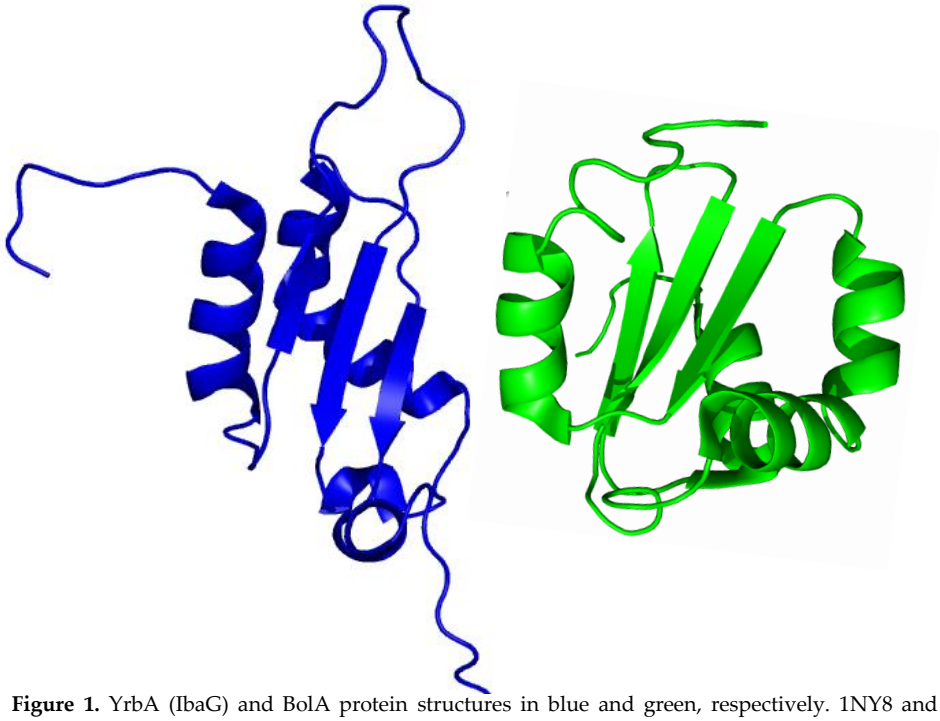


Figure 1. YrbA (IbaG) and BolA protein structures in blue and green, respectively. 1NY8 and 2DHM PDB's from http://www.pdb.org/, cartoons were created using *pymol* evaluation software.

The single *yrbA (*as the double) deletion mutant grow better than the wild type and, in turn, the increased *yrbA* copy number strain decreases both growth and viabilities, in rich neutral medium. The latter plasmid does not produce a morphology phenotype as pMAK580 does, nor does it change *bolA* expression. The reverse is also true although a minimum of BolA seems to be crucial for *yrbA* to be properly transcribed. While most of the *bolA* known phenotypes are not reproduced by *yrbA*, this gene is responsive to acid stress, and was thus named *ibaG*, "induced by acid gene". Moreover we have determined an inversion in the growth curves dynamics upon pH5 acid challenge, where the *ibaG* overexpression strain grew the best while the *ibaG* deletion strain grew the worst, indicating that this gene is involved in resistance and survival against acid stress.

METHODS

Bacterial strains, plasmids and genetic manipulations

The strains and plasmids used in this study are described in Table 1. MG1693 chromosomal DNA was used as a template for polymerase chain reaction (PCR) with *Pfu* polymerase from Fermentas amplification of *yrbA* with surrounding regions using the CLON1 (5'-TGCTGCCATACGTACA GGTG-3') and pCLON2 (5'-GCTGGCTTAGCAGCTTCATTG-3') primers. 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 *AatI* and *PsIt* restriction sites that were separately digested. The 5319 bp plasmid fragment and the PCR reaction digestion were purified with the illustra™ GFX™ PCR DNA and Gel Band Purification Kit from GE Healthcare. Overnight ligation was performed by T4 DNA ligase from Roche to produce the pBGA01 plasmid.

Table 1. Strains and plasmids used in this study.

Strains	Description	Reference or source	Observations
MG1693	thyA715	Bachmann and Low, 1980	background strain
CMA83	MG1693 + pBr325	(this study)	Gibco Brl commercial plasmid
CMA84	MG1693 + pBGA01	(this study)	strain overexpressing *yrbA(ibaG)* from plasmid
CMA10	MG1693 + pMAK580	Santos et al., 1999	overexpressing *bolA* from plasmid
JW3157	MG1655Δ*ibaG*::Kan^r	(Baba et al., 2006)	Keio collection mutant for *yrbA(ibaG)*
JW5060	MG1655Δ*bolA*::Kan^r	(Baba et al., 2006)	Keio collection mutant for *bolA*
CMA85	MG1693 Δ*ibaG*::Kan^r	(this study)	*yrbA(ibaG)* deletion mutant (Keio derived)
CMA86	MG1693 Δ*bolA*::Kan^r	(this study)	*bolA* deletion mutant (Keio derived)
BT340	DH5α+pCP20	(Cherepanov and Wackernagel, 1995)	thermosensitive plasmid expressing recA, to RFFP excise the antibiotic cassette inserted in the genome
CMA87	MG1693 Δ*bolA*	(this study)	*bolA* deletion mutant without the Kanamycin resistance cassette
CMA88	MG1693 Δ*bolA*Δ*ibaG*::Kan^r	(this study)	double *bolA* & *yrbA(ibaG)* deletion mutant
CMA63	BL21+p363	(Freire et al., 2009; Miksch et al., 2005)	BL21 with plasmid encoding for GFP after Multi Cloning Site to evaluate transcription levels of the genes after the promoters inserted
CMA89	MG1693+pRMA02	(this study)	MG1693 with plasmid expressing GFP downstream the *yrbA(ibaG)* promoter
CMA90	MG1693Δ*bolA*::Kan^r +pRMA02	(this study)	*bolA* deletion strain with plasmid expressing GFP downstream the *yrbA(ibaG)* promoter
BL21(DE3)			Novagen commercial strain
CMA50	BL21 (DE3) + pPFA02	(Freire et al., 2009)	BL21(DE3) with plasmid over-expressing (His)6–BolA
CMA91	BL21 (DE3) + pPFA02+pRMA02	(this study)	CMA50 with plasmid expressing GFP downstream the *yrbA(ibaG)* promoter

The *bolA*, *ibaG*, and double *bolA*/*ibaG* deletion mutants were constructed for the MG1693 background using the Keio collection deletant strains kindly provided by Keio University (Baba et al., 2006). Gene transfer was achieved by P1-mediated transduction according to method previously described (Miller, 1972). 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 cured following the published protocol (Datsenko and Wanner, 2000). A plasmid pRMA2 was constructed containing the *gfp* gene encoding for green fluorescent protein under the control of *ibaG* (*yrbA)* promoters, using vector p363 (Miksch and Dobrowolski, 1995). *yrbA* promoter was amplified using primers yrFw (5'–GGGGTACCGCTGCTCCATCTTATCGATC–3') and yrRev (5'–CCATCG ATGGGATCTTCAATCATCAG – 3'). The result fragment was digested with *ClaI* (Fermentas) and cloned directly in p363 digested with the same restriction enzyme. When necessary, strains were transformed with plasmid pCP20 (commercial plasmid), pBr325 (commercial plasmid), pBGA01 (this study), pMAK580 (Aldea et al., 1988), or pRMA02 (this study). Transformations were carried out as previously described (Sambrook, 1989). All plasmids and deletion mutants were confirmed by DNA sequencing at STAB Vida, Portugal (data not shown).

Media, growth conditions, and viability evaluation

Luria broth (LB), M9, and Luria agar (LA) compositions were prepared as described previously (Miller, 1972). When required, the media were supplemented with 0.4 mM thymine, 50 mg/ml chloramphenicol, and 50 mg/ml kanamycin, 0.04% glucose (w/v), (all from Sigma) and 1 mM IPTG (from Apollo Scientifics). For acid challenge strains were started after an overnight growth, and

grown in LB until they reached an OD_{620nm} between 0.4 and 0.5, at which moment all cultures were briefly centrifuged, at about 5500 g for 15 minutes, 4 ºC, to change media into a buffered LB at different pH. Strains were tested against pH 3, 4, and 5 in LB buffered with sodium citrate LB and citric acid, according to previous description (Lin et al., 1995). Optical densities were measured in an Amersham Biosciences Ultrospec®500/1100*pro* spectrophotometer at 620nm, using 10 mm light path *couvettes*. 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 growth curves. Batch cultures were either launched from 16h overnight *inoculi* at 30ºC (for mild growth) or 37 ºC and 100 r.p.m., 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 r.p.m. 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 to grow in the same media for 60 minutes – corresponding to starvation (Starv 1h), and finally re-added with glucose for 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 1000/100µl.

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, according to the growth curve or from cultures growing in M9 at exponential phase (M9 Exp), after one hour 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 (Guinote et al., 2010). The plasmid used for expression of BolA was a pET28a derived pPFA02 (Freire et al., 2009) transformed into a Novagen *E. coli* BL21 (DE3) strain (Table 1). Purification of BolA was performed by histidine affinity chromatography using HiTrap 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 pH 7.4 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 same molarity of BSA control protein in a reference flow cell, used to correct for refractive index changes and non-specific binding (Guinote et al., 2010). The *ibaG (yrbA)* promoter and open reading frame (ORF) were amplified by PCR using yrFw and YrRev (see *Bacterial strains, plasmids and genetic manipulations*) and yrbA3: 5'-

GCCAGCGCAACTTTGCTC-3' and yrbA5: 5'- CCCATAGCTCAAAAGCCG-3' primers, respectively. To create a fragment only containing the ORF, the second PCR product was digested with *NcoI*, and purified with the Nucleic Acid and Protein Purification kit: NucleoSpin®Extract II, from Macherey-Nagel. As a positive control, the promoter sequence of the *mreBCD* operon was used and as a negative control we tested *bolA* open reading frame (ORF) DNA encoding fragment as previously described (Freire et al., 2009). The assays were run at 25 ºC in 20 mM Sodium Phosphate pH 7.4, 1 mM dithiothreitol, and 500mM NaCl buffer as previously described (Guinote et al., 2010). Equilibrium constants were determined using the BIA Evaluation 3.0 software package, according to the fitting model 1:1 Langmuir Binding, and χ^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 points along the growth curve – OD_{620nm}= 0.4, 1.7, and 5 - or after addition of osmotic or acidic stresses: 350mM NaCl (Muffler et al., 1996) or 30% HCl, lowering the pH at 7.2 to 4.4 (Bearson et al., 1997) both at time 0 and 60 minutes. Total RNA was extracted as described (Santos *et al*., 1997). 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 (yrbA)*, in Northern blot experiments spanned the entire transcriptional units and were obtained by PCR, using Taq polymerase (Fermentas) and respectively the primers P2 (5'- CTGTGTTTCAATCT TTAAATCAGTAAACT -3') and X9 (5'- CCAGACAAAACAAACGGCCCG-3'), and 3 (5'- GCCAGCGCAACTTTGCTC-3') and 5 (5'-CCCATAGCTCAAA AGCCG-3'), in the case of DNA probes, or P2 and X9_T7 (5'- G TTTTTTTTAATACGACTCACTATAGGCCAGACAAAACAAAACGGCCCG-3') and 3 and 5_T7 (5'- GTTTTTTTTAATACGACTCACTATAGGCCCATAGC TCAAAAGCCG-3'), in the case of RNA probes. 3 µl of pUC Mix Marker, 8

(Fermentas) was labeled with [δ-32P]-dATP using PNK and 100 times diluted into the RNA samples loading buffer, and 7.5 μl were run simultaneously to the samples to determine their molecular weight. All radioactive labels were cleaned in G-50 columns from GE healthcare.

Northern blot and hybridization

Samples containing 15 μg of total RNA were dissolved in 90% formamide, 0.01 M EDTA pH 7.0, 1 mg/ml xylene cyanol, 1 mg/ml bromophenol blue buffer (Sambrook *et al*., 1989), heated for 5 min at 100ºC for denaturation, and incubated for 10 min on ice. Total RNA samples were electrophoresed on a 6% denaturing polyacrylamide gel and transferred to a nylon membrane (Biodyne A from PALL) 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-32P]-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 essentially as described by Sambrook *et al*. (1989). Filters were visualized using the PhosphoImager System from Molecular Dynamics.

Transcription evaluation by p363 derived plasmid

Transcription evaluation was analyzed using *gfp* as reporter gene using the p363-derived vector (Miksch and Dobrowolski, 1995), pRMA02 (see *Bacterial strains, plasmids and genetic manipulations*). BL21 + pPFA02 + pRMA02 was grown at 120rpm 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 done as described before (Freire et al., 2009). Results are showed 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.

RESULTS AND DISCUSSION

IbaG is an E. coli BolA homologue

In *Escherichia coli*, when the *bolA* morphogene is overexpressed, cells become round, and cells multiplication is affected. Nevertheless, the deletion of this gene does not show any significant phenotype in rich growth medium. When a gene is lost or mutated in a way that the correspondent protein function is compromised, it tends to be at least partially replaced by others that share some characteristics. A search was performed using NCBI public resources and an uncharacterized gene was found to have a strong protein similarity with BolA, particularly considering the shared *bolA*/*yrbA* domain. This *ibaG* gene is expressed counterclockwise at the 71.87 minutes of the *E. coli* genome, upstream of *murA* and downstream of an operon of five genes (Fig. 2). Although the expression of the surrounding genes is co-directional *ibaG* is not predicted to be co-expressed with the upstream operon, but only its single gene expressed from its own σ70-predicted single promoter region as evaluated by the REGULON DB 6.7: Gene Form. The upstream genes to *ibaG: mlaBCDEF* (plus *mlaA*) compose the Mla pathway, an ABC transport system whose function seems to prevent phospholipidic accumulation in the outer leaflet of the Gram-negative bacteria outer membrane (OM), thus contributing to the preservation of the OM lipid asymmetry (Malinverni and Silhavy, 2009).

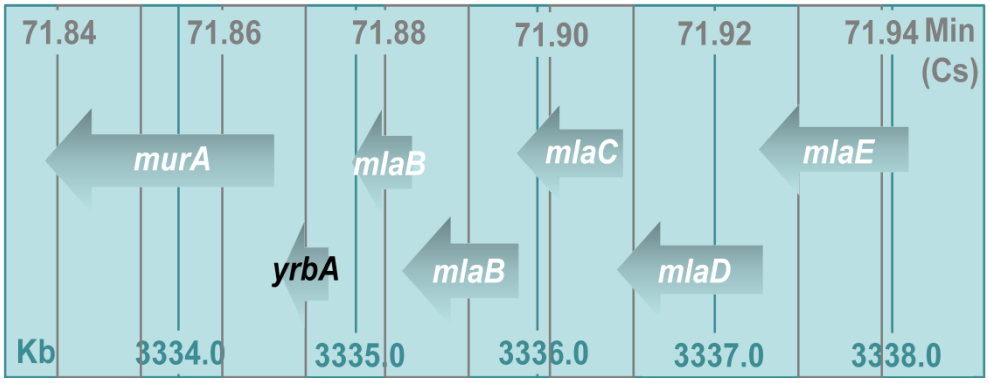


Figure 2. *ibaG* (*yrbA)* gene proximal genetic map from *Escherichia coli*, adapted from Ecogene public server.

The downstream gene of this series is the essential *murA* gene, that encodes for the UDP-N-acetylglucosamine enolpyruvyl transferase, which synthesizes peptidoglycan precursors from N-acetylglucosamine acid and phosphoenoylpyruvate (Brown et al., 1995; Herring and Blattner, 2004; Marquardt et al., 1992). 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 toxics or injuries. The genes that occur in the proximity as well as the sequence homology that this gene has with *bolA*, increased the interest in studying *yrbA*.

ibaG is not an essential gene and both single and double bolA/ibaG deletions improve E. coli growth

Similarly to what had been appreciated for *bolA*, the single deletion of *ibaG (yrbA)* did not prove unviable for *E. coli* cells (Baba et al., 2006). The double deletion Δ*bolA*Δ*yrbA* mutant was expected to be lethal, but such supposition proved to be wrong. After removing the kanamycin resistance cassette introduced in the place of the *bolA* gene, by pCP20 transformation and cure, and deletion of the *ibaG* gene with simultaneous insertion of the region coding for kanamycin resistance, both regions were sequenced to confirm the disruption of the respective genes (data not shown). Finally, growth, viability, and morphological analysis were

performed to check the phenotypical effects due to the absence of these proteins. At least in the optimal growth conditions used, and contrarily to what had been anticipated, the deletion mutants grow similarly or better than the background strain MG1693. In fact, although differences are small, both Δ*yrbA* and Δ*bolA*Δ*yrbA* deletion mutants grow about 1.2 times faster (evaluated by the exponential phase rate of growth) and reach higher OD than the wild type (Fig. 3A). These results are confirmed by the number of colony forming units obtained for the lag, early exponential, mid exponential, and late exponential/early stationary phases of growth evaluated (Figure 3B). The most significant difference among cultures viability refers to the background MG1693 strain which forms about half or even less number of colonies than any of the deletion mutants. In the beginning of the growth curve differences among strains are minimal, and until early stationary phase (inclusive), all cultures reveal a viability increment, as expected. However, there is a transitory decrease in 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 *ΔbolA* to the *ΔibaG* and the *ΔbolAΔibaG* strains, where the latter is the most significantly distinct. As for the late stationary phase the MG1693 viabilities are similar to early stationary phase and quite stable along the entire 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. The decrease in growth potential in exponential phase may reduce the exhaustion of important resources or prevent some catabolites to be created and released to the media, thus favouring the population maintenance in stationary phase (Figure 3B). Finally, morphology assessment was made for background and all deletion strains at the same time points where viability was evaluated. All strains evolved similarly (Figure 4A).

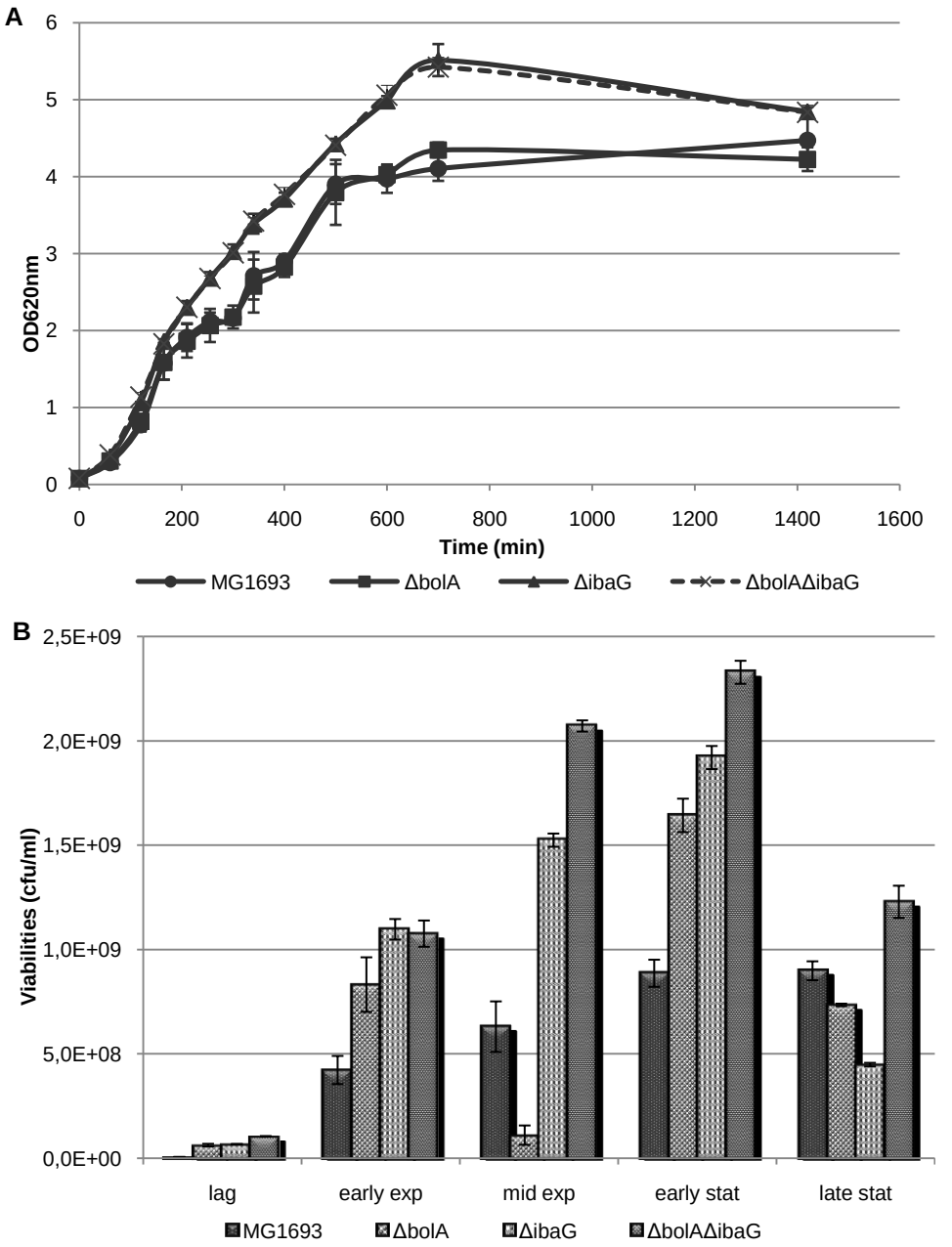


Figure 3. Growth curves and viabilities depending on the absence of *bolA, ibaG*, or both genes. **A.** Optical Density measurements at 620nm for determination of growth curves in LB media supplemented at 37ºC and 120 r.p.m. according to the strains: MG1693, MG1693Δ*bolA*, MG1693Δ*yrbA* and MG1693Δ*bolAyrbA*. **B.** Viability evaluated by number of colony forming units (see Materials and Methods) in properly supplemented LA plates for the same strains and conditions.

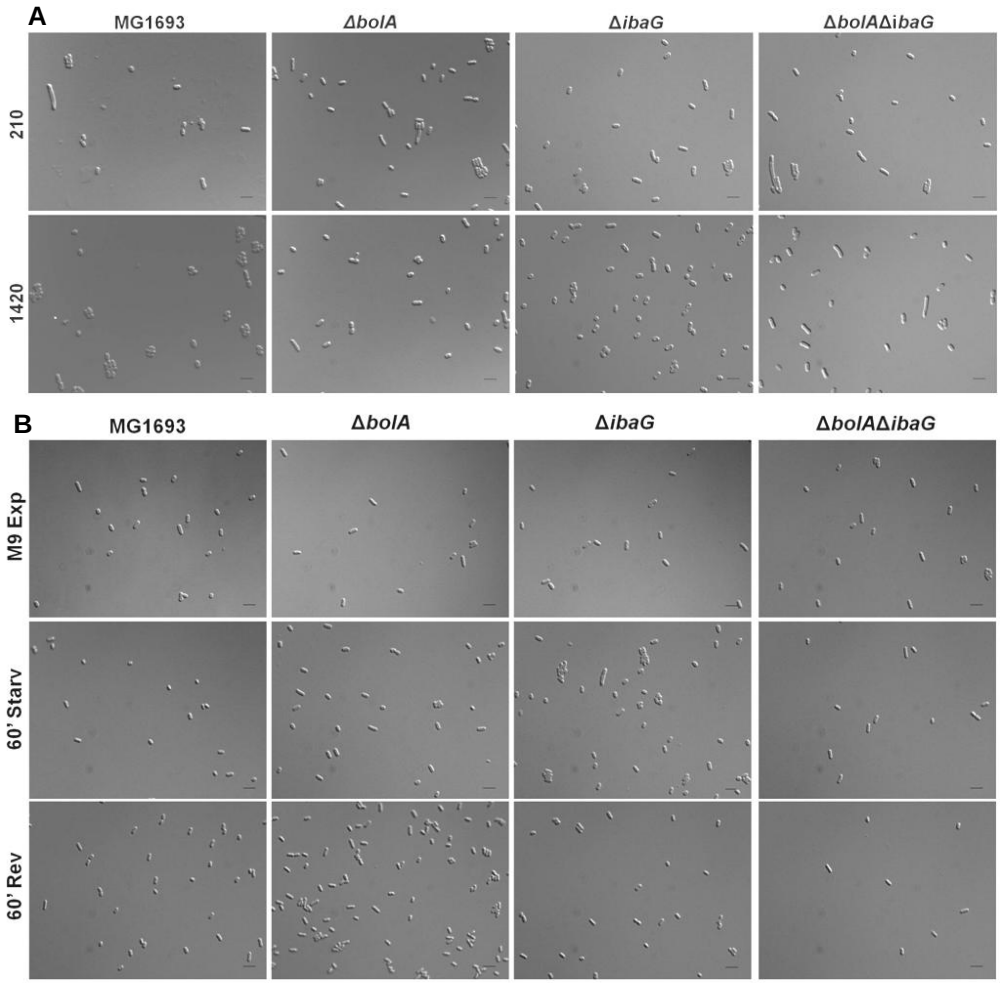


Figure 4. Differential Interference Contrast microscopy photographs, obtained using a DMRA microscope (Leica) for the strains MG1693, MG1693Δ*bolA*, MG1693Δ*yrbA*, and MG1693Δ*bolA*Δ*yrbA*. **A.** Evaluation at time points 60, 120, 340, 700, and 1420 min of the LB growth curves; **B.** Evaluation at time points M9 grown exponential, one hour after induced starvation, and fifteen minutes after reversion (see Materials and Methods). The dark bar represents 5 μm.

Given that *bolA* and *ibaG* respectively are and may be involved in the cell protection against stresses, morphologies were also analysed in poor or 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).

Morphologies are kept similar to wild type in the presence of an increased ibaG copy number

Even though the deletion of *bolA* does not reflect in cells' morphologies and growth curve, the same is not true for the increased *bolA* copy number expressed after its own promoters in a pBR325 derived plasmid, pMAK580 (Aldea et al., 1988; Guinote et al., 2011). To further pursue the characterization of *ibaG* we have cloned it preceded of the respective promoter, in an attempt to create a similar construct to plasmid pMAK580, pBGA01. Growth curves, viability assessment and morphological study were implemented afterwards. The MG1693 strain transformed with pBr325 was used as an additional control or background strain when evaluating the effects of the pMAK580 and pBGA01 plasmids. Similarly to what is reported for MG1693, this control strain showed not to change growth dynamics according to the conditions imposed to the starter culture (Figure 5A). Oppositely, MG1693 transformed with pMAK580 strongly depends on the conditions cultures were exposed before *inoculum* into the new growth media (Guinote et al., 2011; Guinote et al., 2010). According to this, conditions were preset with a 3 to 4 hours growth at 30ºC with aeration, for *bolA* overexpression not to be so deleterious, growing to a maximum OD of about 3.5 before dilution into the fresh medium. To evaluate YrbA effects usual overnight pre-growth at 37ºC with aeration was performed, so that any phenotypes associated with this protein expression would be determined. The enhanced expression of *ibaG* using pBGA01 showed a deleterious effect as evaluated in the growth curve (Figure 5A), nevertheless they were significantly less notable than pMAK580 effects under the same conditions (Guinote et al., 2011). The viabilities were evaluated in the different strains (Figure 5B). The presence of pBr325 does not change viability results except at mid exponential phase. The number of dividing cells is similar to MG1693 background strain for all the other time points evaluated. As for the pre-growth conditions, MG1693 strain diluted after reaching

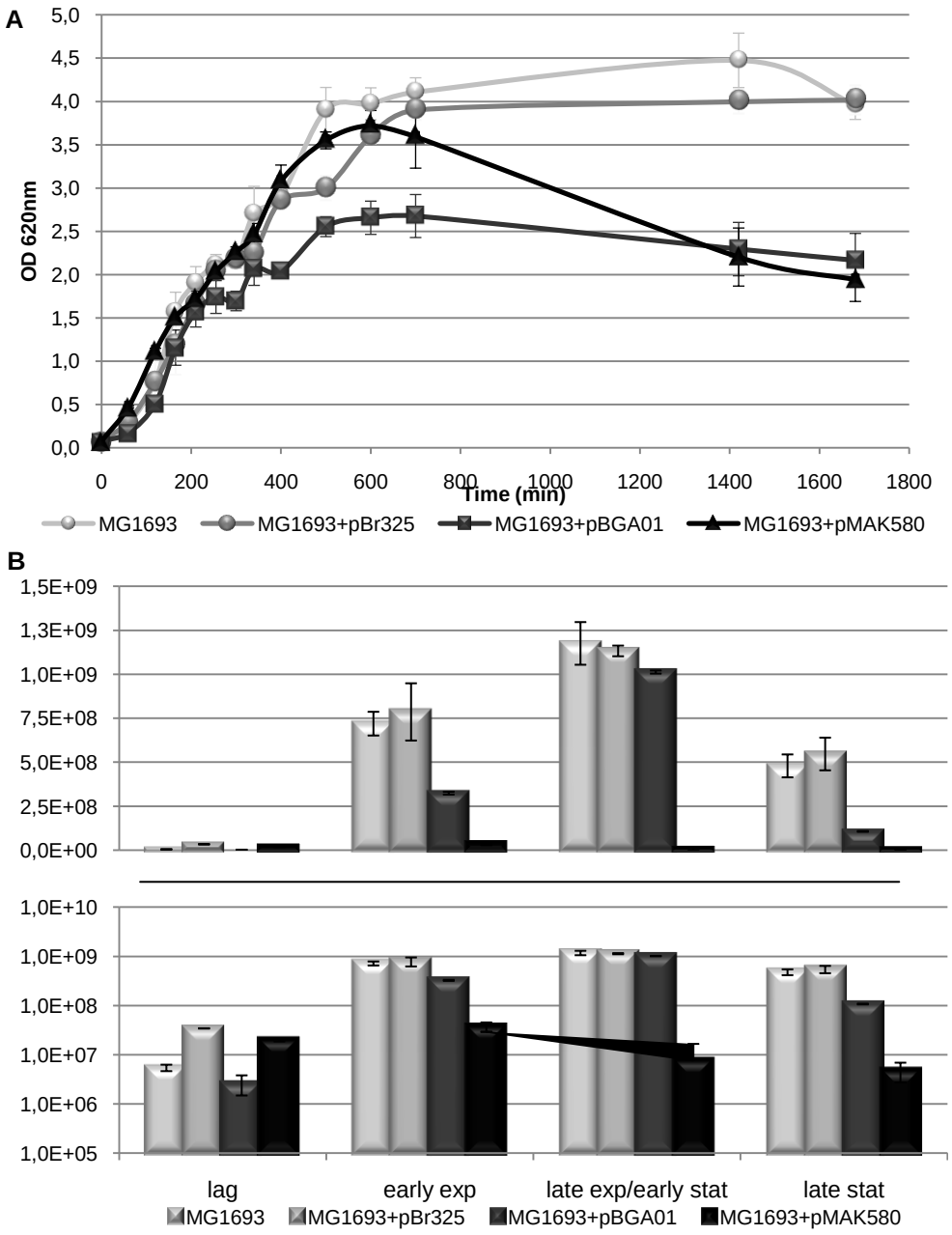


Figure 5. Growth curves and viabilities induced by the excess of *bolA, ibaG*, or the control plasmid. **A.** Optical Density measurements at 620nm for determination of growth curves in LB media supplemented at 37ºC and 120 r.p.m. according to the strains: MG1693, MG1693+pMAK580, MG1693+pBGA01, and MG1693+pBr325. **B.** Viability evaluated by number of colony forming units (see Materials and Methods) in LA plates for the same strains and conditions.

stationary phase had slightly reduced cell counts comparing to the one diluted from exponential phase as expected. Similarly to what was observed in the growth curves, viability results, show that overexpression of *ibaG* by pBGA01 plasmid was detrimental in general, with the sole exception on the transition to stationary phase. This plasmid significantly reduces colony counts. In pMAK580 transformed strain, the viabilities were always quite low and more or less constant (Fig. 5B, logarithmic scale) (Guinote et al., 2011). Since *bolA* is a morphogene, and its effects are absolutely visible by microscopy evaluation when it is overexpressed by pMAK580, this methodology was used to evaluate the plasmid pBGA01 phenotype. Microscopy did not help the characterization of the function of YrbA, since no changes were observed in the presence of the later plasmid (Fig. 6). An eventual stress reaction by this gene, as described above, was evaluated growing all these strains in M9 and sugar depleting the cultures. Only pMAK580 transformed strain showed spheres with the conditions imposed. The pBGA01 strain behaved similarly to the wild type MG1693.

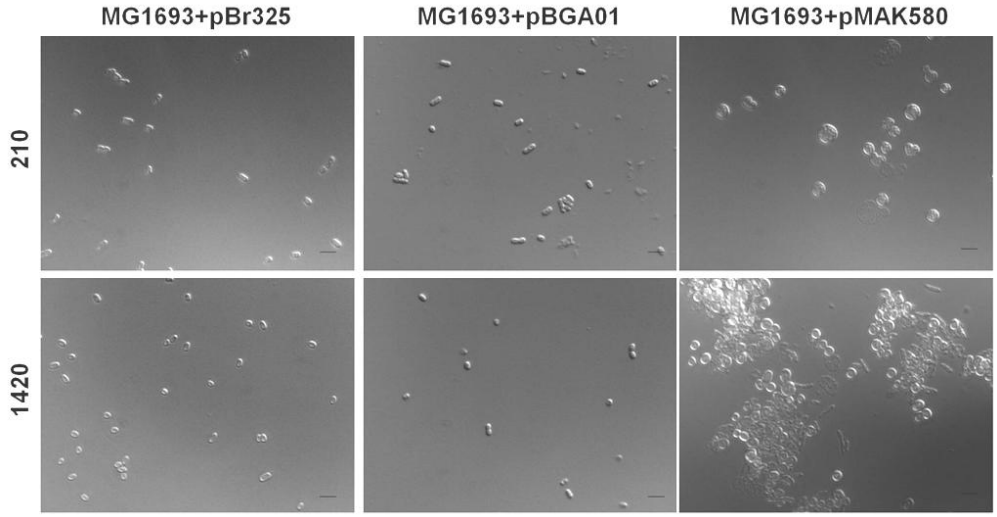


Figure 6. Differential Interference Contrast microscopy photographs, obtained using a DMRA microscope (Leica) for the strains MG1693, MG1693+pMAK580, MG1693+pBGA01, and MG1693+pBr325, at time points 60, 120, 340, 700, and 1420 min of the LB growth curves. The dark bar represents 5 μm.

ibaG is not induced or repressed by BolA but seems to require its presence for transcription

According to the previous results *ibaG* and *bolA* seemed not to complement but override effects, thus seeming to concur in parallel and not a single pathway. Moreover it was possible to observe that BolA overexpression has a stronger phenotype than YrbA. Since BolA was shown to interact with the promoter regions of *mreB*, *dacA* and *dacC*, Surface Plasmon Resonance (SPR) experiments were used to test the ability of that protein to recognize and interact with the *ibaG* promoter and open reading frame (ORF) regions. The results show that BolA does not interact directly with *ibaG* (Table 2). Therefore, BolA was established not to act as a direct transcriptional regulator for *ibaG*. Nevertheless induction of indirect transcriptional changes were evaluated by means of pRMA02, a p363 based plasmid, where GFP is expressed according to the upstream promoter activity (Fig.7). This methodology allows transcription activity of any cloned promoters to be measured by determination of fluorescence. The *ibaG* promoter did not reveal to be either activated or repressed by increased BolA levels (induced by addition of IPTG to the BolA overexpressing pET28a derived plasmid, pPFA02 (Freire et al., 2009)).

Table 2. BolA binding affinity for yrbA 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^0 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.

	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

Nevertheless, GFP measured transcription levels were halved when *bolA* was repressed (by addition of glucose to the same system) (Fig. 7). Minimal levels of BolA, thus seemed to be required for the proper transcriptional activity of this promoter. This would provide relevance to the *bolAp*2 weak constitutive promoter, which maintains low levels of *bolA* expression.

To confirm this hypothesis wild type and *bolA* deletion strains were also transformed with pRMA02 plasmid and the transcription activity of *iba*G promoter was evaluated in both backgrounds. Validation was achieved given that the transcription was decreased 30% in mid exponential phase and 70% in stationary phase. When *bolA* is poorly expressed the difference of *iba*G transcription between the background and the *bolA* deletion strain is much lower than in stationary phase, when the expression of *bolA* is physiologically more significant (Fig. 7).

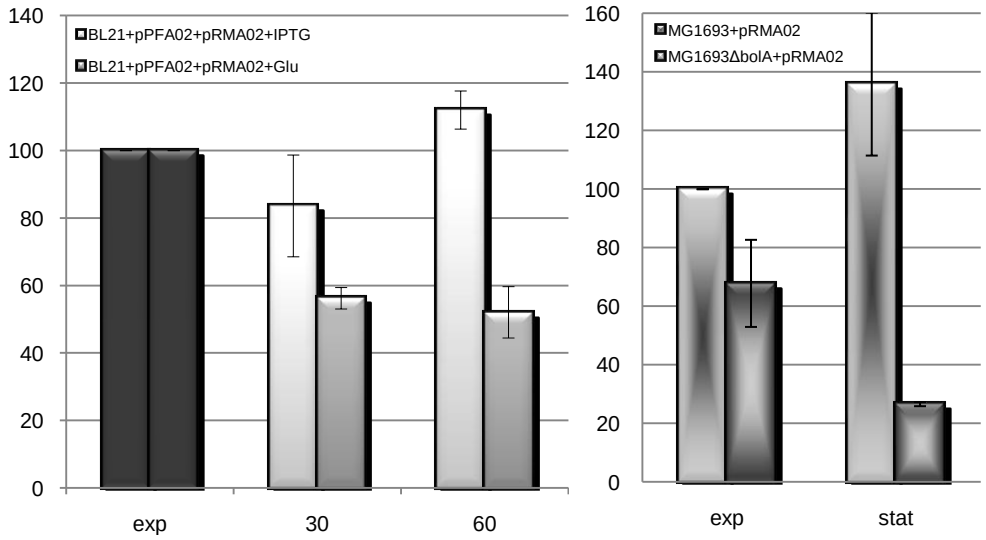


Figure 7. yrbA transcription evaluation by determination of pRMA2 GFP fluorescence per cell at OD 0.5, and 30, and 60 minutes after addition of either glucose or IPTG to BL21´pPFA02+pRMA02 (left) and at mid exponential and late stationary phase of MG1693+pRMA02 and isogenic *bolA* deletant (right). The averages of GFP fluorescence per cell (by EF-Tu quantification) were determined as a percentage towards the exhibited at exponential 0.4 OD.

ibaG (yrbA) 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. As expected *bolA* transcript was absent in the deletion strain, and similarly expressed 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. *BolA* mRNA levels were only increased in the strain transformed with pMAK580, the plasmid that overexpresses *bolA*. As a result, we could confirm that *ibaG* higher mRNA levels did not influence the *bolA* transcripts (Fig. 8).

When we used the *ibaG* probe for equivalent membranes it was possible to see that, in the single copy genome, *ibaG* does not present sufficient levels to be detected (at least with 15ug total RNA per lane used in this study). Nevertheless the expression of the latter gene could be acknowledged when strains were transformed with pBGA01 plasmid, where *ibaG* is expressed after its own promoter but is present in more copies. As it is a pBr325 derived plasmid, it is present until five times more in stationary phase. According to this, if *ibaG* mRNA levels increased at that phase, more than five times higher levels should be detected. However, the transcripts of *ibaG* presented the lower levels exactly in stationary phase, which means that the gene is basically absent at this developmental phase of *E. coli* (Fig. 9). The highest expression of this gene occurs at mid exponential phase, with correspondence to the start of the increase in *bolA* mRNA levels. Two different stresses were checked, namely, the osmotic and the acid stress. It was observed that, when cells were challenged with hidrochlorous acid stress, *ibaG* increases its levels and assumes a completely different expression pattern (Fig. 9). When osmotic stress is imposed *ibaG* expression is not shut down, but the mRNA seems to become degraded into multiple fragments. *ibaG* shares with *bolA* the ability to strongly respond to stress.

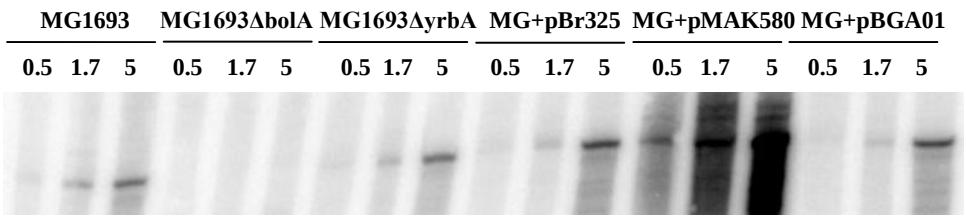


Fig. 8. Northern Blot analysis of the expression of *bolA* mRNA in MG1693, MG1693ΔbolA, MG1693ΔyrbA, MG1693+pBr325, MG1693+pMAK580, and MG1693+pBGA01, respectively, at OD 0.5, 1.7, and 5, corresponding to early exponential, mid exponential, and stationary phases.

Fig. 9. MG1693+pBGA01, yrbA mRNA at OD 0.5, 1.7_control for stresses, after 1 hour of HCl and NaCl challenges, and OD 5, corresponding to early exponential, and mid exponential phase, acid (pH4.4) and osmotic stresses (NaCl 0.35 M) imposition for one hour, and stationary phase. This transcript could not be detected in any of the other strains (data not shown).

Former yrbA, now ibaG, standing for induced by acid gene, favours growth in mild acid media

This gene may be involved in the survival or growth enhancement in conditions of mild acid. All the strains tested stopped growing as they did not further increase their ODs after the pH of the medium was modified to 3 or 4 (data not shown). Nevertheless, when pH 7 LB was switched to pH 5 LB, the strain that was overexpressing *ibaG* could grow better than the wild type and, conversely, the deletion strain was more sensitive (Fig. 10). Between 180 and 240 minutes after the stress challenge the strains are basically overimposed in OD and gradually split with inverted pattern to what happened at the pH 7 LB growth. Therefore this *ibaG* does not seem to be responsible for resistance against strong

acid pressures (pH 3 and 4) but is certainly involved in tolerance against mild acid

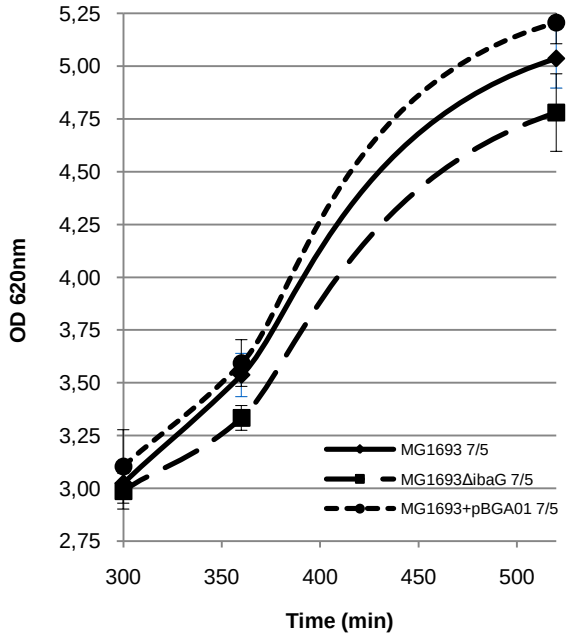


Fig. 10. Growth behaviour of MG1693, MG1693ΔyrbA, and MG1693+pBGA01, determined for pH5 acid stress challenge induced after OD 0.4/0.5 was reached by all cultures. MG1696+pBr325 strain grew similarly to the deletion strain (data not shown).

environments (pH 5). In a similar situation to *bolA*, *ibaG* mRNA is strongly increased in exponential phase, upon acid stress imposition, and could additionally be demonstrated to contribute to *E. coli* tolerance against acid stress.

FINAL REMARKS

The BolA protein homologues are widely distributed in nature, and even represented in several copies or homologue genes within the same organisms, with the exception of Gram-positive bacteria. BolA has been predicted to be a reductase that interacts with a mono-thiol glutaredoxin (which would provide it reducing equivalents to the evolutionarily conserved cysteine's loss). Accordingly, BolA was proposed to be responsible for the reduction of organic peroxides (Huynen et al., 2005). Additionally, an attempt to characterize a BolA/YrbA homologue of *Pseudomonas fluorescens*, revealed a mild phenotype

related to the sulphur metabolism. Nevertheless the "*bolA*" mutation could only be reverted transforming the strain with *bolA* homologue added of the two upstream genes that encode for a putative sulphurtransferase and a dissulfideisomerase, respectively (Koch and Nybroe, 2006). So far, the *bolA* yeast homologue was the sole protein that revealed common growth, division and resistance/survival characteristics to the *E. coli bolA* (Kim et al., 2002). In this report, we have shown YrbA to be a BolA homologue protein, with significant aminoacid sequence similarity and structural fold maintenance. The initial idea that this homologue could be substituting for *bolA,* when the latter was deleted from *E. coli* genome, and in consequence preventing a more evident phenotype to be presented was disproven. All attempts to characterize *ibaG* gene and protein similarly to BolA proved unfruitful, since it did not produce any of the morphological or growth dynamics' associated changes, either by means of the deletion or overexpression methodologies. In the case of the YrbA overexpression, placid decrease in the growth rate, in the maximum OD reached by the culture, and in cell counts were determined, with exception of the transition from exponential to stationary phase. Such reduction was not as pronounced as the reported for BolA overexpression plasmid, pMAK580, nevertheless. Moreover, even though the single Δ*ibaG* and double ΔΔ*bolA*/*ibaG* deletion mutants demonstrated improved and additive growth curves and viabilities, the cells' morphologies did not show any differences to the background strain independently on the media tested. Also the strains transformed with the control pBr325 and the pBGA01 *ibaG* increased expression plasmids, did not change morphologies in any of the growth and stress conditions evaluated.

BolA assumes a protective role of the cell in stress conditions, when its expression levels are induced. This may be the only commonality between these two *E. coli* proteins apart from the aminoacidic and folding similitude, which has

conferred them the status of homologues. IbaG was demonstrated to be strongly expressed as a reaction to acidic stress, in a process that is simultaneously characterized by what seems to be the maturation of the biggest transcript into strictly two others different than the shorter fragments exhibited by this gene in good growth conditions. Given the protein structural similarity to BolA and increased expression in response to stress, but also the additive features in their derived growth, and viabilities, it is feasible that *ibaG* hydrochloric acid response represents a parallel to *bolA* mechanism of cell defence. The parallel and not singular pathways or mechanisms yet to characterize would justify the inability of one gene to complement the other but both absences to be synergetic. Although morphology or expression pattern did not recognize YrbA to mimic or overlap BolA, this gene has been proven to share with BolA more than overall structure, namely the response against acid stress and increasing tolerance upon those conditions.

ACKNOWLEDGEMENTS

We would like to thank Keio University (Baba et al., 2006) 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 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.

REFERENCES

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 9:3787-94.

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 170:5169-76.

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 8:3923-31.

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. Mol Syst Biol 2:2006 0008.

Bearson, S., B. Bearson, and J.W. Foster. 1997. Acid stress responses in enterobacteria. FEMS Microbiol Lett 147:173-80.

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 177:4194-7.

Cherepanov, P.P., and W. Wackernagel. 1995. Gene disruption in Escherichia coli: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. Gene 158:9-14.

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 97:6640-5.

Freire, P., R.N. Moreira, and C.M. Arraiano. 2009. BolA inhibits cell elongation and regulates MreB expression levels. J Mol Biol 385:1345-51.

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 88:341-6.

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 260:106-11.

Guinote, I.B., P. Freire, and C.M. Arraiano. 2011. BolA can contribute to dormancy, protecting cells against external stresses in E. coli: is bolA a new persister gene? Microbiology (*in preparation)*.

Guinote, I.B., R.G. Matos, P. Freire, and C.M. Arraiano. 2010. BolA affects growth and binds to the promoters of Penicillin-Binding Proteins 5 and 6

regulating their expression. Journal of Microbiology and Biotechnology:*in press*.

Herring, C.D., and F.R. Blattner. 2004. Conditional lethal amber mutations in essential Escherichia coli genes. J Bacteriol 186:2673-81.

Huynen, M.A., C.A. Spronk, T. Gabaldon, and B. Snel. 2005. Combining data from genomes, Y2H and 3D structure indicates that BolA is a reductase interacting with a glutaredoxin. FEBS Lett 579:591-6.

Jasiecki, J., and G. Wegrzyn. 2003. Growth-rate dependent RNA polyadenylation in Escherichia coli. EMBO Rep 4:172-7.

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 13:545-8.

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 14:425-30.

Koch, B., and O. Nybroe. 2006. Initial characterization of a bolA homologue from Pseudomonas fluorescens indicates different roles for BolA-like proteins in P. fluorescens and Escherichia coli. FEMS Microbiol Lett 262:48-56.

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 173:4474-81.

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 177:4097-104.

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 106:8009-14.

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 174:5748-52.

Martinez-Antonio, A., S.C. Janga, and D. Thieffry. 2008. Functional organisation of Escherichia coli transcriptional regulatory network. J Mol Biol 381:238-47.

Miksch, G., F. Bettenworth, K. Friehs, and E. Flaschel. 2005. The sequence upstream of the -10 consensus sequence modulates the strength and induction time of stationary-phase promoters in Escherichia coli. Appl Microbiol Biotechnol 69:312-20.

Miller, J.H. 1972. Experiments in Molecular Genetics Cold
Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

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 178:1607-13.

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.

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 32:789-98.

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 157:31-8.

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 45:1729-40.

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 60:177-88.

Vieira, H.L., P. Freire, and C.M. Arraiano. 2004. Effect of Escherichia coli morphogene bolA on biofilms. Appl Environ Microbiol 70:5682-4.

Chapter 5

GENERAL DISCUSSION

BolA is an *E. coli* protein which causes round morphology when overexpressed (Aldea et al., 1988). The amounts of this protein in the cell are usually reduced and tightly controlled. The gene *bolA* is transcribed from the weak constitutive promoter P2, under the control of sigma70 (RpoD) and the main gearbox promoter P1 under the control of both sigmaS (RpoS) and sigma70 (Friedman et al., 2005; Nguyen et al., 1993), is transcribed mainly in conditions where sigmaS levels are high, like stationary phase (Aldea et al., 1989; Lange and Hengge-Aronis, 1991; Loewen and Hengge-Aronis, 1994) or during the exponential phase of growth, when triggered in response to several forms of stress (Santos et al., 1999). Northern blot analysis showed that when *bolA* transcription is induced the mRNA transcript is also degraded 3 times slower (Freire et al., 2006a; Santos et al., 2006). The complementary regulation, at the level of the *bolA* mRNA stability, enables the cell to respond faster to the need of turning on or off genes. The consonance between transcriptional and post-transcriptional controls is expected when gene products have global effects over cellular regulation and are only required for specific periods, as seems to be the case of *bolA*.

This morphogene seems to be involved in the cell division control (Aldea et al., 1989; Freire et al., 2009) induces biofilm formation (Vieira et al., 2004) and affects cellular permeability (Freire et al., 2006b). Moreover *bolA* was shown not only to have its mRNA and protein levels affected by the cells growth rate, but its overexpression also strongly reduces the growth of cultures and viability counts. The reduction of metabolism, cell shape modifications, and increased ability to endure imposition of external stresses, desynchronization of population growth, dependency on stationary phase, and even more importantly the potency to stimulate the production of biofilms, strongly suggested *bolA* to be a persister gene. Increased expectations arise from the idea that this protein can be modulating the response of microorganisms against stresses imposed by their

hosts therefore making bacteria less susceptible to treatment. The study of persister genes may also enlighten understanding of molecular mechanisms underlying diseases such as cancer where cells, attempting to protect themselves against all kinds of aggressions, gain the ability to change their metabolism, shape, and even aggregation behaviour, and also inducing changes in their surroundings.

BolA was also proposed to act as a transcriptional regulator. It was shown that it interacts with the promoter of *mreB* (Freire et al., 2009). MreB is a cytosqueleton protein that spirals along the cells maintaining their rod shape. BolA decreases the levels of MreB which leads to the loss of its ability to polymerize properly, while maintaining the spirals. After being established that BolA increases the levels of the penicillin binding proteins PBP5 and PBP6 and the β-lactamase AmpC mRNA (Santos et al., 2002), it was confirmed that BolA can also interact with the *dacA* and *dacC* promoters and in that way directly regulate their transcripts (Guinote et al., 2010). Although overlapping of PBP5 and PBP6 functions is imperfect, some substitution effect was confirmed in the sense that the BolA overexpression related growth and morphology phenotypes are only prevented when both DD_carboxypeptidases PBP5 and PBP6 are depleted. PBP5 and PBP6 share 65% homology and the peptidoglycan remodeling type of activity, although the second presents about 4 times less specific activity (Amanuma and Strominger, 1980). In addition, overexpression of either of them can reverse the effects of a specific temperature-sensitive allele of PBP3, again showing to perform similar functions *in vivo* (Begg et al., 1990). However, this two proteins do not functionally overlap on the basis of their carboxypeptidase activities, or on their penicillin binding abilities (Ghosh and Young, 2003); and overexpression of one or the other leads to completely different results: high levels of PBP5 originate spheres out of rods (Markiewicz et al., 1982) and the same

is not seen for PBP6 (van der Linden et al., 1992). On the other hand, the latter was suggested to stabilize by cross-linking (van der Linden et al., 1992) the peptidoglycan of nongrowing cells (Glauner and Holtje, 1990) and aminoacid starved cells (Goodell and Tomasz, 1980), in analogy to the *Bacillus subtilis* sporulation-specific PBP5a (PBP5*) (Buchanan and Ling, 1992; Todd et al., 1985). Thus a strong relation of a BolA target PBP5 and bacterial shape alterations and PBP6 with change in the characteristics of murein and increasing cell wall resistance, could be determined. Although there is not just one function or phenotype associated to BolA we can already establish a strong relation of this protein with the cell wall function and regulation. Additionally, the cross-linking PBP5 and PBP6 enzymes may be involved in some kind of control of population or host´s perception of the presence of bacteria by differential cross-linking and due to the release of D-Ala-Ala dipeptides, when those proteins are active. This may be another way by which BolA helps bacteria avoid the immune system/external stresses imposition.

Finally, BolA has possible homologues throughout the living world apart from gram-positive bacteria. This absence may be due to the fact that this gene appeared only after evolutional divergence between this two types of bacteria or simply because its targets do not exist or are not functionally relevant in Gram-positive species. The major difference between Gram-positive and Gram-negative bacteria resides on the cell wall. Gram-negative bacteria have an outer membrane after a thin peptidoglycan layer, while Gram-positive have a unique inner membrane followed by a thick murein layer in which teichoic acids are arranged creating a negative charge. Apart from this structural difference, gram-positive species are also the only bacteria that are able to sporulate (although spores can also be found among plants, algae, fungi and some protozoans). This stable dormant or resting forms occur in their life cycle to enhance their survival under

adverse conditions. This fact further substantiates the theory of BolA as a persister protein, preventing cell death in response to negative stimuli given that Gram-negative bacteria lack the ability to sporulate.

Additionally to the existence of predicted BolA homologue proteins in other organisms, one was determined to have high similarity level in *E. coli*, that was designated as *yrbA*, and was renamed as *ibaG*. This protein shares a common *bolA*/*yrbA* domain, also harbouring the Helix-Turn-Helix motif with propensity for nucleic acid binding. The substitution of BolA by IbaG could thus explain why the deletion of the first gene was not lethal or even detrimental for growth (except in minimal medium). A regulatory (eventually inter-regulatory) mechanism could exist to equilibrate both mRNA/protein levels. However, the double deletion mutant constructed during this doctoral work did not behave as predicted. On the contrary, deleting either *ibaG* or *bolA*/*ibaG* has surprisingly revealed to be an asset for the cultures growth and viabilities in optimal growth conditions. Even more, overexpressing *ibaG* under its own promoters (by pBGA01 plasmid) lead to a decrease in growth and viabilities, corroborating the previous result. Interestingly enough, *ibaG* is encoded in the chromosome neighbourhood of a series of genes related to the peptidoglycan synthesis and differential composition of the outer membrane. This relates to the exchanges with the environment as well as adherence and communication, all of which aspects are related to BolA. Even though *ibaG* regulation is not similar to *bolA* and it is not directly affected by the respective protein, nor it presents a similar phenotype to the homologue, *ibaG* was proven to be related to stress and enhance the multiplicative potential of cells in adverse low pH conditions. We have thus named this gene as induced by acid gene, *ibaG*.

All of these data emphasize the present interest in better characterize the expression of the almost ubiquitous *bolA* gene and its homologues in *E. coli*, and higher organisms. In this way we may not only advance in the characterization of global protective regulation in microorganisms but eventually also provide new tools or data and insights for developmental and survival processes that are known to occur and to be strongly affected by the environmental conditions.

REFERENCES

http://www.britannica.com/EBchecked/topic/48203/bacteria/272362/Sporulation?anchor=ref955422.

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 170:5169-76.

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 8:3923-31.

Amanuma, H., and J.L. Strominger. 1980. Purification and properties of penicillin-binding proteins 5 and 6 from Escherichia coli membranes. J Biol Chem 255:11173-80.

Begg, K.J., A. Takasuga, D.H. Edwards, S.J. Dewar, B.G. Spratt, H. Adachi, T. Ohta, H. Matsuzawa, and W.D. Donachie. 1990. The balance between different peptidoglycan precursors determines whether Escherichia coli cells will elongate or divide. J Bacteriol 172:6697-703.

Buchanan, C.E., and M.L. Ling. 1992. Isolation and sequence analysis of dacB, which encodes a sporulation-specific penicillin-binding protein in Bacillus subtilis. J Bacteriol 174:1717-25.

Freire, P., R.N. Moreira, and C.M. Arraiano. 2009. BolA inhibits cell elongation and regulates MreB expression levels. J Mol Biol 385:1345-51.

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 88:341-6.

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 260:106-11.

Friedman, N., S. Vardi, M. Ronen, U. Alon, and J. Stavans. 2005. Precise temporal modulation in the response of the SOS DNA repair network in individual bacteria. PLoS Biol 3:e238.

Ghosh, A.S., and K.D. Young. 2003. Sequences near the active site in chimeric penicillin binding proteins 5 and 6 affect uniform morphology of Escherichia coli. J Bacteriol 185:2178-86.

Glauner, B., and J.V. Holtje. 1990. Growth pattern of the murein sacculus of Escherichia coli. J Biol Chem 265:18988-96.

Goodell, W., and A. Tomasz. 1980. Alteration of Escherichia coli murein during amino acid starvation. J Bacteriol 144:1009-16.

Guinote, I.B., R.G. Matos, P. Freire, and C.M. Arraiano. 2010. BolA affects growth and binds to the promoters of Penicillin-Binding Proteins 5 and 6 regulating their expression. Journal of Microbiology and Biotechnology:*in press*.

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 173:4474-81.

Loewen, P.C., and R. Hengge-Aronis. 1994. The role of the sigma factor sigma S (KatF) in bacterial global regulation. Annu Rev Microbiol 48:53-80.

Markiewicz, Z., J.K. Broome-Smith, U. Schwarz, and B.G. Spratt. 1982. Spherical E. coli due to elevated levels of D-alanine carboxypeptidase. Nature 297:702-4.

Nguyen, L.H., D.B. Jensen, N.E. Thompson, D.R. Gentry, and R.R. Burgess. 1993. In vitro functional characterization of overproduced Escherichia coli katF/rpoS gene product. Biochemistry 32:11112-7.

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 32:789-98.

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 45:1729-40.

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 60:177-88.

Todd, J.A., E.J. Bone, and D.J. Ellar. 1985. The sporulation-specific penicillin-binding protein 5a from Bacillus subtilis is a DD-carboxypeptidase in vitro. Biochem J 230:825-8.

van der Linden, M.P., L. de Haan, M.A. Hoyer, and W. Keck. 1992. Possible role of Escherichia coli penicillin-binding protein 6 in stabilization of stationary-phase peptidoglycan. J Bacteriol 174:7572-8.

Vieira, H.L., P. Freire, and C.M. Arraiano. 2004. Effect of Escherichia coli morphogene bolA on biofilms. Appl Environ Microbiol 70:5682-4.

Chapter 6

PERSPECTIVES

In extension to the work until now developed around the idea of BolA as a persistence protein, we think it will be important to evaluate its potential in growth dynamics and survival against a series of antibiotics. Although minimal inhibitory concentrations (MIC) are expected to be unchanged by persister proteins we think it will be good to determine them. For such we will initially screen qualitatively the reaction of our strains to different antibiotics, measuring the halo of growth inhibition created around disks of discrete amounts of antibiotic.

Since purification of his-tagged BolA has been done and optimized, we hope to obtain good, potent and specific antibodies, a tool that will open several new doors in the study of this protein. The quantification of the protein levels will be possible and we expect to compare them with the levels of the transcripts and to confirm the premise of a global concerted regulation of BolA in the cell.

To further study the regulation of this morphogene, both *bolA1p* and *bolA2p* mRNA levels and stability will be further investigated by steady state and decay evaluation of the transcripts by Northern Blot Analysis in RNase II, III and E mutants, and eventually in double Hfq/RNase(s) mutants. In all the strains the protein levels are expected to be compared with the transcripts data. As the transcript has a quite long 3' tail after the open reading frame, we would like to investigate if *bolA* is also controlled by small RNAs.

Surface Plasmon Resonance analysis using a sensorchip with BolA immobilized may provide the means to analyse specific interactions with different cell extracts or specific molecules to evaluate how they are modulated, and eventually recover the partners for identification, through mass spectrometry.

Another goal is to define where and by which mechanisms BolA and the proteins whose expression it regulates are acting on: division itself, elongation machinery, and/or definition of the division plate. To evaluate if the cell wall if splitting isn't exactly at the middle of the cell as it is predicted, and if the proper patchwork of

the mature *vs.* new murein is being affected by BolA, the peptidoglycan turn-over in the several strains could be determined fluorescently labelling cell walls. Moreover, the use of strains or plasmids where some cytosqueletal elements or penicillin-binding proteins are fused with several forms of eGFP making them observable by optic microscopy through the emission of fluorescence at different wavelengths, would certainly shed light into this temporal, organizational and localized concerted mechanisms.

The determination of where BolA is being expressed/acting inside the cell and of the structures/metabolism proteins/effectors it interacts with, eventually allowed with eGFP fused BolA. This could later on help in the definition of the protein's functions and complexes formed. The simultaneous microscopic study with DAPI for the chromatin organization would possibly help understanding how the protein expression can eventually be related to the *bolA* position in the chromosome or by some DNA differential compaction at its location in different stages of growth and conditions in several mutants, as well as the protein's action over other genes expression.

If resolution is not enough to visualize specific BolA localization(s), labelling the anti-BolA antibodies with gold (Au) probes to hybridize the bacteria, observing them by electronic transmission microscopy, would improve visualization, even though only providing picture of dynamic processes. This later procedure would also help determining the degree of chromatin compaction according to the BolA levels in different strains.

The *bolA* expression is concomitant with the accumulation of stationary-phase protein Dps, compacting the nucleoid and eventually regulating gene expression profiles, also conferring cells increased resistance against a variety of stresses (Almiron et al., 1992; Altuvia et al., 1994; Boylan et al., 2003; Choi et al., 2000; Martinez and Kolter, 1997; Nair and Finkel, 2004; Stephani et al., 2003). The growth-phase dependent intrinsic MdtEF multidrug efflux systems additionally

confer tolerance to toxics (Kobayashi et al., 2006). It could also be interesting to observe the Dps and eventually MdtEF mRNA levels at least in wt *vs*. *bolA* deletion strain.

On the other hand, considering the predicted action of BolA protein as scavenger for organic peroxides reduction (Huynen et al., 2005) and that Dps, also presents metal chelating and ferroxidase activities it could be interesting to determine the cell's redox potential, ROS accumulation, eventual ferrous and ferric iron distribution/deposition and perhaps the expression metals export proteins in the studied strains. Microscopy evaluation would certainly provide information both beautiful and meaningful.

To acknowledge what is determining the size of the cell (increase due to accumulating solutes followed by water, enhancing turgor pressure, or the augmentation of cell wall resistance in certain regions after alteration of cell wall composition) and the cell shape (with changes in the "2" dimensions of the cell, or only one: length or radius), it should be interesting to analyse cell wall and internal cell solutes composition, and determine BolA related levels.

Finally, functional DNA microarrays from a *bolA* deletion mutant based on the MG1655 type strain *versus* the isogenic pMAK580 overexpression, are now being compared. Precious information is expected to be retrieved and integrated with all the previous results. Based in these data we expect to develop an integrated model for BolA. It is anticipated that this can provide knowledge to implement biotechnological tools for differential modulation of gene expression and ultimately control cell growth and division.

REFERENCES

Almiron, M., A.J. Link, D. Furlong, and R. Kolter. 1992. A novel DNA-binding protein with regulatory and protective roles in starved Escherichia coli. Genes Dev 6:2646-54.

Altuvia, S., M. Almiron, G. Huisman, R. Kolter, and G. Storz. 1994. The dps promoter is activated by OxyR during growth and by IHF and sigma S in stationary phase. Mol Microbiol 13:265-72.

Boylan, J.A., J.E. Posey, and F.C. Gherardini. 2003. Borrelia oxidative stress response regulator, BosR: a distinctive Zn-dependent transcriptional activator. Proc Natl Acad Sci U S A 100:11684-9.

Choi, S.H., D.J. Baumler, and C.W. Kaspar. 2000. Contribution of dps to acid stress tolerance and oxidative stress tolerance in Escherichia coli O157:H7. Appl Environ Microbiol 66:3911-6.

Huynen, M.A., C.A. Spronk, T. Gabaldon, and B. Snel. 2005. Combining data from genomes, Y2H and 3D structure indicates that BolA is a reductase interacting with a glutaredoxin. FEBS Lett 579:591-6.

Kobayashi, A., H. Hirakawa, T. Hirata, K. Nishino, and A. Yamaguchi. 2006. Growth phase-dependent expression of drug exporters in Escherichia coli and its contribution to drug tolerance. J Bacteriol 188:5693-703.

Martinez, A., and R. Kolter. 1997. Protection of DNA during oxidative stress by the nonspecific DNA-binding protein Dps. J Bacteriol 179:5188-94.

Nair, S., and S.E. Finkel. 2004. Dps protects cells against multiple stresses during stationary phase. J Bacteriol 186:4192-8.

Stephani, K., D. Weichart, and R. Hengge. 2003. Dynamic control of Dps protein levels by ClpXP and ClpAP proteases in Escherichia coli. Mol Microbiol 49:1605-14.

Chapter 7

APPENDIX

INDEX

Appendix I

Host Factor HFQ

and the regulation of bolA

INDEX

ABSTRACT

Both Hfq and BolA proteins seem to play a general role in stress response regulation in *E. coli*, changing the expression of outer membrane proteins, histone-like proteins, and thus DNA condensation and accessibility. Additionally, they are partially sigma S regulator and regulated, respectively. Hfq is a ribonucleic acids chaperone and facilitator of nucleic acid-sRNA-protein interaction, thus inter-regulating their levels, while BolA is a transcriptional regulator and they may have complementary functions. The two respond to different stresses, particularly sugar presence/depletion, and both mRNAs are stabilized upon entry into stationary-phase. Their absence or excessive presence, respectively, changes the central carbon flux, production of extracellular products, biofilm formation, and eventually virulence. In this work Hfq was confirmed to be involved in the post-transcriptional control of *bolA* and to be essential for the steady-state levels of this transcript. Growth curves, viability analysis and morphology evaluation, further documented the importance of Hfq presence for BolA cellular effects to be achieved.

INTRODUCTION

The induction of genes such as *bolA* has been so far mostly accounted for at the level of transcription. However, our results show that there is an integrated answer between transcription and degradation. Under conditions where *bolA* is more transcribed its message is less degraded and when it is less transcribed the RNA decay is much faster (Santos et al., 1999). The main *E. coli* ribonucleases involved in the degradation of mRNA are ribonuclease II (RNase II), polynucleotide phosphorylase (PNPase), ribonuclease III (RNase III), and ribonuclease E (RNase E). Mutants deficient in these ribonucleases were used to determine which was responsible for the *bolA* mRNA decay. Even though the decay of this transcript is really fast in conditions where it is not needed (when stresses cease to exist), none of the most common RNases seems to participate in the degradation of this messenger RNA, with the exception of RNase III, according to what has been previously described (Freire et al., 2006; Santos et al., 1997).

Hfq is a 15 KDa, nucleic acid-binding protein, originally identified as an *Escherichia coli* host factor required for RNA phage Q (beta) replication. It is a pleiotropic post-transcriptional regulator of gene expression, modulating both mRNA stability and translational activity, due to its interactions with several small non-coding RNA species (sRNAs) (Takada et al., 1997) (Arluison et al., 2006). Hfq is apparently auto-repressed at the translational level and positively regulated at the transcriptional level by the alarmone ppGpp, the stringent response alarmone, and by the general stress response DksA (Sharma and Payne, 2006).

Hfq is responsible for increasing growth rate and culture yields, and presents several other effects in the cell: enhances DNA supercoiling in the stationary phase, reduces cell size, confers resistance to stresses, reduces cellular oxidation state, facilitates biofilm formation and nitrogen fixation, and allows lambda

phage plaque formation. It strongly influences virulence in pathogenic bacteria, affecting σ^S-independent cell motility, membrane composition, adhesion, invasion, ability to replicate in macrophages and to secrete effector proteins (Tsui et al., 1994) (Sharma and Payne, 2006) (Ansong et al., 2009; Sittka et al., 2007).

Hfq harbours two independent RNA binding surfaces: the Proximal Site, which binds sRNAs and mRNAs; and the Distal Site, which binds poly(A) tails and was shown to interact with Polyadenylation Polymerase I (PAP I), stimulating its activity (Le Derout et al., 2003) (Ziolkowska et al., 2006), and Polynucleotide Phosphorylase (PNPase) and RNase E, forming complexes involved in the mRNA decay pathway (Mikulecky et al., 2004) (Mohanty et al., 2004) (Brennan and Link, 2007). Nevertheless, RNA degradation by the 3'-5' exoribonucleases PNPase and RNase II at oligo(A) tails and RNase E hydrolysis of the interhelical regions, was shown to be prevented by Hfq binding, *in vitro*, suggesting it modulates the sensitivity of RNA to ribonucleases in the cell (Folichon et al., 2003). Large subsets of additional proteins were identified co-purifying with Hfq: ribosomal proteins, RNA degradation and processing proteins, chaperones, transcription proteins, RNA modification proteins, and the Lon ATP-dependent protease (Wilusz and Wilusz, 2005).

Stationary phase *rpoS* as well as *bolA1p* and *dps* mRNAs are all polyadenylated, eventually preventing them from degradation, and thus enhancing their stability (Cao and Sarkar, 1997) (Cunning et al., 1998). Besides protecting mRNAs with poly(A) tails, Hfq also seems to confer protection to the sRNAs since the putative RNase E recognition sites (A/U-rich sequences and adjacent stem-loop structures) are coincident with the Hfq binding sites, preventing sRNAs from being degraded by endonucleolytic attack, without their targets attached (Moll et al., 2003b).

On the other hand, pairing with the specific mRNA, it can block translation initiation and promote their degradation (Arluison et al., 2007) (Geissmann and

Touati, 2004) (Udekwu et al., 2005). Hfq binds the sRNA bases that overlap the Shine-Dalgarno sequence of the specific target's mRNA and accelerates the rate of duplex formation between them, forming a ribonucleoprotein complex with RNase E/degradosome, which degrades both RNAs, promoting an RNase E-dependent gene silencing and translation inhibition (Aiba, 2007) (Kawamoto et al., 2005). Hfq furthermore affects the mRNA's accessibility to transcription, interfering with the ribosome binding and start codon accessibility, even after they are no longer bound, given that the induced mRNA's structural changes are not reversible (Moll et al., 2003a). A Hfq null mutant enhances *ftsZ* mRNA, as well as protein levels, in stationary-phase, leading to high frequency mini-cells, especially in poor-media (Takada et al., 1999).

Hfq seems to play a general role in stress response regulation in E. coli, repressing the σ^E mediated envelope stress response (outer membrane proteins); and also induces the σ^{32} -mediated cytoplasmic stress response due to decreasing DnaK expression (Guisbert et al., 2007). Hfq moreover regulates DNA repair pathways in a σ^S-dependent (MutH and MutS) and independent way (MutS, in the exponential as well as stationary phase of growth) the different mechanisms eventually accounting for differential adjustment to different stresses (Tsui et al., 1997). Response to glucose-P accumulation is sensed by the SgrR transcription factor that activates the SgrS sRNA negatively regulating, by Hfq/RNaseE & degradosome complex, the stability and translation of the ptsG mRNA encoding for the major *E. coli* glucose transporter, limiting the toxic accumulation (Vanderpool, 2007). On a reverse regulation mechanism, CsrA binds specifically to the Shine-Dalgarno sequence of *hfq* leader transcript, inhibiting Hfq synthesis by competitively blocking ribosome binding. Hfq mRNA is nevertheless stabilized upon entry into stationary-phase by a CsrA-independent mechanism (Babitzke and Romeo, 2007) (Baker et al., 2007). Depending on the particular organism, the Csr (carbon storage regulation - or Rsm) system, which consists of

CsrA, a homodimeric RNA binding protein, two noncoding small RNAs, CsrB and CsrC, that function as CsrA antagonists, and CsrD, that targets CsrB and CsrC for degradation by RNase E, participate in global regulatory circuits that control central carbon flux, the production of extracellular products, cell motility, biofilm formation, quorum sensing and/or pathogenesis.

Hfq is a global regulator of stress response, partially through σ^S dependent induction of genes, but its role, mainly in virulence, is not exclusively attributable to σ^S. This molecule seems to be mandatory for the virulence of several pathogenic bacteria, namely *Salmonella typhimurium*, *Vibrio cholerae*, *Listeria pneumophyla*, *Shigella flexneri*, *Yersinia enterocolitica*, *Brucella abortus*, and *Pseudomonas aeruginosa* (Brown and Elliott, 1996) (Ding et al., 2004) (McNealy et al., 2005) (Sharma and Payne, 2006) (Sonnleitner et al., 2003).

Pairing data on BolA and Hfq, interaction and regulation were postulated to occur. Thus we have purposed to analyze Hfq as a possible factor involved in the post-transcriptional control of *bolA* and understand how it works in consonance with transcription, to keep in balance the global regulation of this gene. Growth curves viability analysis and morphology evaluation, were performed and mRNA decay of both the wild type and *hfq* deletion strains was analysed to evaluate the stability of the *bol*A mRNA message, depending on Hfq. The presence of Hfq was demonstrated to be essential for BolA effects to occur, and interestingly in its absence the "excess" of BolA led to unexpected filaments or at least long rods that eventually diverged in the poles. This effect was exacerbated when growth media was supplemented with glucose, that is known to destabilize *hfq*. That supplement also induced dramatic changes on all strains growth, faster at first but strongly reduced as evaluated by the maximum OD attained by the cultures; also morphologies were slightly affected, enhanced thickness of the wild type cells, and inducing "filamentation" and the up come of bulges in the long rods of CMA10 overexpressing BolA strain.

METHODS

Bacterial strains, plasmids and genetic manipulations

The strains used in this study are described in Table 1. When necessary, strains were transformed with plasmid pMAK580 (Aldea et al., 1988) containing *bolA* under regulation of its own promoters. Transformations were carried out as previously described (Sambrook, 1989).

Table 1. Strains used in this study

Strains	Description	Reference or source	Observations
MG1693	thyA715	Bachmann and Low, 1980	background strain
CMA10	MG1693 + *bolA*$^+$	Santos et al., 1999	MG1693 overexpressing *bolA* from pMAK580
CMA29	thyA715 Δhfq:Kan^r	Freire, 2005	hfq deletion mutant based on MG1693
CMA14	CMA29::Kan^r +*bolA*$^+$	(this dissertation)	CMA29 overexpressing *bolA* from pMAK580

Media, growth conditions and viabilities

Luria broth (LB) and Luria agar (LA) were prepared as described previously (Miller, 1972). When required, the media were supplemented with 0.4 mM thymine, 0.4% glucose, 50 mg/ml chloramphenicol, and 50 mg/ml kanamycin (all from Sigma). Optical densities were measured in an Amersham Biosciences Ultrospec®500/1100*pro* spectrophotometer at 620nm, using 10 mm light path *couvettes*. 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 growth curves. Batch cultures were either launched directly from previous LA plate grown colonies or from overnight *inoculi* at 37 ºC and 100 r.p.m., which were diluted to an optical density of 0.08 measured at 620 nm (OD_{620}). Cultures were grown aerobically at 37 ºC and 120 r.p.m. For evaluation of viability, the samples were processed in LB

serial dilutions, and 100 µl 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 1000/100µl.

Microscope preparations

To observe the effect of BolA overexpression and *hfq* deletion 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, according to the growth curve or from cultures growing in M9 at exponential phase (M9 Exp), after one hour 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.

RNA extraction and probe preparation

Culture samples were taken at the desired points along the growth curve or after addition of osmotic or acidic stresses: 350mM NaCl (Muffler et al., 1996) or 30% HCl, lowering the pH at 7.2 to 4.4 (Bearson et al., 1997) both at time 0 and 60 minutes. Total RNA was extracted as described (Santos *et al*., 1997). 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 *yrbA*, in Northern blot experiments spanned the entire transcriptional units and were obtained by PCR, using Taq polymerase from Fermentas and primers P2 (5'-CTGTGTTTCAATCTTTAAATCAGTAAACT -3') and X9 (5'- CCAGACAAAACA AAACGGCCCG-3'), and 3 (5'- GCCAGCGCAACTTTGCTC-3') and 5 (5'-CCCAT AGCTCAAAAGCCG-3'), respectively.

Northern blot and hybridization

Samples containing 15 mg of total RNA were dissolved in 90% formamide, 0.01 M EDTA pH 7.0, 1 mg/ml xylene cyanol, and 1 mg/ml bromophenol blue buffer (Sambrook *et al*., 1989), heated for 5 min at 100ºC for denaturation, and incubated for 10 min on ice. Total RNA samples were electrophoresed on a 6% denaturing polyacrylamide gel and transferred to a nylon membrane (Biodyne A from PALL) 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 probe radiolabelled with [α-32P]-dCTP, using the Multiprime DNA labelling system from Amersham. Probe hybridization with PerfectHyb™Plus Hybridization Buffer 1x, was carried out at 42ºC. Amersham Hybond™-N⁺ nitrocellulose membranes optimized for nucleic acid transfer from GE Healthcare were hybridized and washed essentially as described by Sambrook *et al*. (1989). Filters were visualized using the PhosphoImager System from Molecular Dynamics.

RESULTS AND DISCUSSION

Glucose supplementation enhances rate but reduces overall growth

The *bolA1p* transcript has been recognized to be positively regulated by RNase III (Freire et al., 2006; Santos et al., 1997). Under carbon starvation *bolA2p* transcript is processed to a fragment of the size of *bolA1p* (Santos, 2000). RNase III is involved in this cleavage since in an RNase III deletion mutant only 50% of this transcript is processed. In the absence of Hfq about half of this transcript is also not cleaved. The influence of *hfq* on *bolA* was evaluated by means of an *hfq* deletion strain. Given that usual BolA experiments involve addition and removal of glucose to M9 medium, and that this sugar has strong influence on bacteria regulation, namely through Hfq (Morita et al., 2008; Wadler and Vanderpool, 2009), LB was added of this sugar (over the strains requirements) to see if glucose is responsible for side effects.

When LB media was supplemented with glucose, all strains presented a faster growth rate in exponential phase; conversely, it induced cultures to stop growing sooner and reaching lower maximum ODs. This latter outcome, if considered present, was practically unappreciated in *hfq* deletion strains (about 8% difference, comparing to 60% difference recorded for MG1693 background strain and the isogenic *bolA* deletion strain).

As for MG1693 transformed with pMAK580 strain (CMA10) after an overnight liquid growth this wild type transformed culture would be reluctant on growing, and only after a long (and not always similar) period could restart growth. At the time the work was being performed this was a true "mystery" but it was finally understood that the initial levels of *bolA* are quite important in defining the subsequent growth pattern (see previous chapters 2 and 3). This phenomenon was at the time so incomprehensible that older stocks and newly transformed strains were created and tested for growth and morphologies, to ensure that results were real and accurate. All strains tested had the same phenotype, confirming the results.

Viability assays confirm that growth inhibition by glucose in later phases of bacterial growth is due to catabolites accumulation in the medium

Addition of glucose induced faster growth rates. This growth acceleration was predicted since this sugar is highly energetic, stimulating growth and accelerating metabolism, eventually inducing higher respiration rates and leading to an increase in the cell's oxidative levels. According to this, adding glucose does not only enhance growth but also "ages cells", what may explain the decrease in growth yields.

On the other hand, glucose has been shown to repress the formation of TCA (citric acid) cycle enzymes, namely 2-ketoglutarate dehydrogenase and isocitrate dehydrogenase synthesis, and of cytochrome synthesis in complex media, which

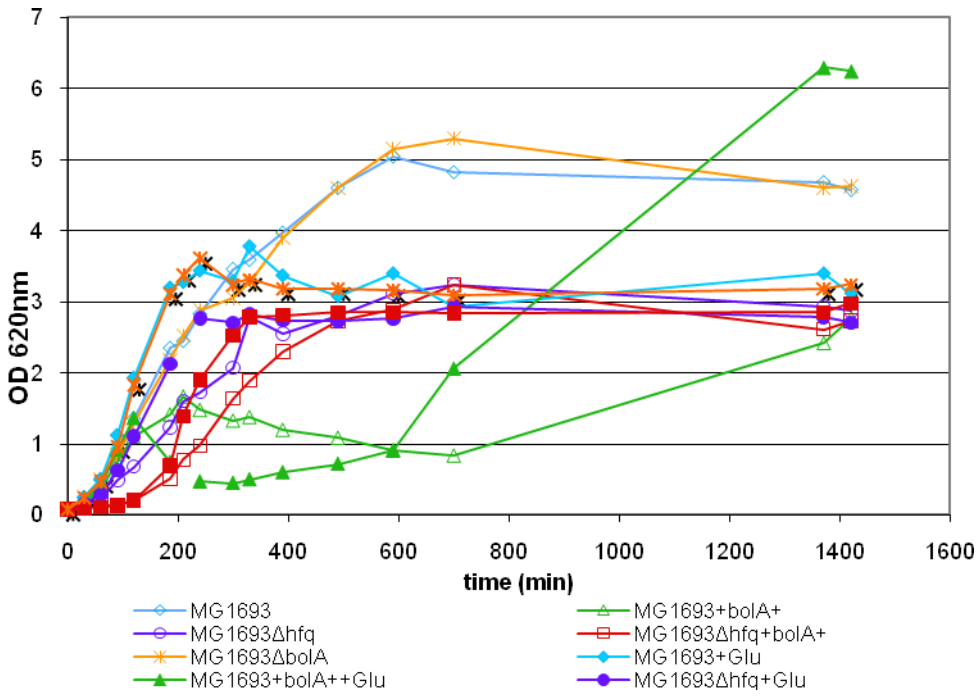


Figure 1. Representative optical density measurements at 620nm. Growth curves in LB media supplemented according to the strains requirements, with or without additional glucose, at 120 r.p.m., 37 ºC; glucose added strains are represented by filled symbols.

is explained by enough ATP being available from the EMP (glycolytic) pathway to minimize the role of the TCA cycle and respiration in energy production. In this sense, not only competing sugars pathways but also oxidative phosphorylation were shown to be subjected to catabolite repression (Horst W. Doelle, 1982). Considering this, the cultures may reach stationary phase sooner due to the wear out of glucose in the medium and consequent reduction of growth rate while cells adapt sugar uptake and catabolism, and due to less energetic efficiency then on (Bruckner and Titgemeyer, 2002). This reduction of growth yied may also be due to the accumulation of toxic products after glucose fast catabolism or to the exhaustion of may other growth-limiting nutrient like any aminoacid, vitamine or growth factor (Pirt, 1967).

Finally, it could be due to a homeostatic equilibrium of cells with the medium. The higher osmolarity of the medium could be responsible for the shrinking of the cells due to some water loss, reflecting on the ability of cells to restrain light path, reducing the OD recorded. To evaluate both causes and real effects on division, viability measurements were performed (Fig. 2).

As expected all strains' colony counts improve along the growth curve, and those where glucose is added tend to present more viable cells than in the absence of this supplement to the medium. In fact, the viabilities determined for the wild type strain did not reflect the growth curves data. They show higher cell counts when glucose is added to the medium, arguing in favor of the hypothesis that the accumulation of "toxic" byproducts is responsible for stalling growth. Given that cultures are diluted into fresh liquid media and cultured on fresh solid media, any toxic released for environment (growth media) would be substituted, enabling the cells to express their growth potential in the absence of the inhibitors (Fig. 2).

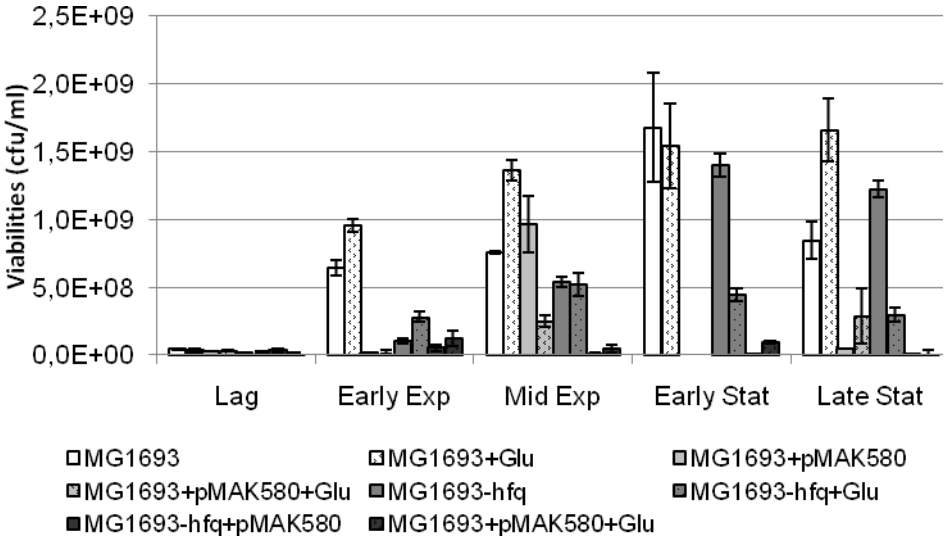


Figure 2. Viability analysis of f glucose on *hfq* mutant strains, at lag, early and mid exponential, early and late stationary phases of growth – average and standard deviations from a minimum of three independent repetitions are presented.

As for the *hfq* deletion strain, the increased growth due to glucose supplementation detected in the ODs was also present in the cell counts until mid exponential phase but not after, similarly to what was referred in the growth curve. There is a significant increment in the number of dividing cells in the latter stages of the growth curve. That may be due to an opposite mechanism to the supplementation or to Hfq not being necessary at those developmental stages of bacterial cultures (Fig. 1 and 2).

Presenting reduced growth in the initial stages of media adaptation this *hfq* deletion may spare (nutritional) resources or avoid the formation of catabolic toxics that would inhibit growth upon accumulation. This strain would therefore have an increased ability to multiply in latter stages, as it is possible to observe in the number of colony forming units, determined for early and late stationary phases. This process may be underappreciated by OD measurements due to cell's sizes induced by the deletion itself. The *hfq* deletant strain has its cell numbers strongly restrained in the beginning of the growth curve when the presence of this protein may be more important for cell development. As time progresses, the viability of this strain is not so impaired, getting to override the wild type strain in late stationary phase, where cell turnover is usually strongly reduced, as regulation of mRNAs, sRNAs and proteins are kept to a minimal function.

The presence of pMAK580 does not seem as injurious for the *hfq* deletant as for the wild type strain in the growth curves but that is not so obvious in the viabilities evaluation. Cell counts are the same for the single deletant and the transformed derived strain in the beginning of growth, but once mid exponential phase is reached it becomes about two orders of magnitude less represented or even four in late stationary phase. Eventually this could not be easily accessed by absorbance measurements due to the shape changes induced by BolA and/or the absence of Hfq, which also strongly affects cells' dimensions.

Hfq & BolA effects are already foreseen in colony growth

Another interesting aspect to be retained from the viability studies is that colonies have differential aspect/dimensions according to the strains: Hfq mutants are the smallest, the wild types significantly larger, and interestingly the CMA29+pMAK580 have intermediate size (Fig. 3). In CMA10 the *bolA* overexpression may also induce a mild increase in the colonies dimensions but differences are not obvious. Cell morphologies were accessed by Differencial Interference Contrast (DIC) microscopy.

Hfq deletion averts BolA overexpression induced spherical morphologies, and instead creates tripolar filaments; less upon glucose addition

Glucose addition revealed to change dimensions of cells, rather than shape. Moreover it induced cells to condense their DNA into reduced compacted regions, usually at one or both poles. In those conditions, DNA compaction was determined from early exponential phase on, while in glucose nonadded medium this phenomenon only occurred at late stationary phase, for part of the population. All cells except those of the wild type strain increased about twice, both in length and radius. The strains that were transformed with BolA overexpressing plasmid additionally gained the ability to form filaments at 340 minutes and forward time points. The expected small cells that characterize the absence of Hfq were detected for the *hfq* single deletant strain grown in LB medium, but such phenotype could unexpectedly be reverted by supplementation with glucose. This chaperone strongly influences cells for glucose uptake and metabolism by the interaction with the global regulator of carbon source metabolism *CprA* (Lucchetti-Miganeh et al., 2008) and/or *SgrS*, the sRNA that destabilizes the glucose permease *ptsG* mRNA (Maki et al., 2008; Vanderpool and Gottesman, 2004).

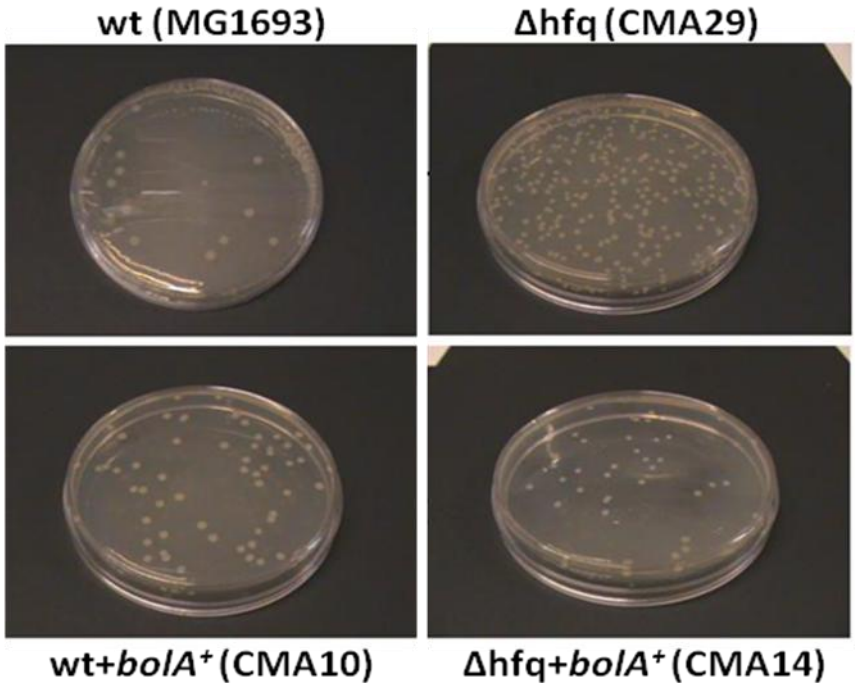


Figure. 3. LA plated with MG1693 (wt), CMA29 (Δ*hfq*) and each strain transformed with pMAK580 (+*bolA*⁺) after 30 minutes of inoculate growth, incubated at 37ºC for 24 hours.

Eventually, the increased availability of glucose as energy source reduces the impact of Hfq absence in what relates to cellular morphologies. This could justify the similarity of morphologies between the wild type and deletant strains, when with glucose. BolA effects of overexpression also increase phenotypes upon glucose addition since the size of the cells is considerably increased. Some CMA10 cells(wild type transformed with pMAK580), at least after 700 minutes, become huge and worm-like, differentiating two poles at one extremity, while others remain symmetrical and present a huge central swelling where the cell nucleoid localizes. Although BolA increased expression induces changes in both wild type and *hfq* deletion strains it is absolutely doubtless that the effects are strongly attenuated in the *hfq* deletion strain. Although small spheres and rare longer or Y cells are formed in the CMA14 strain, it mainly shows regular sized rods (Fig. 4).

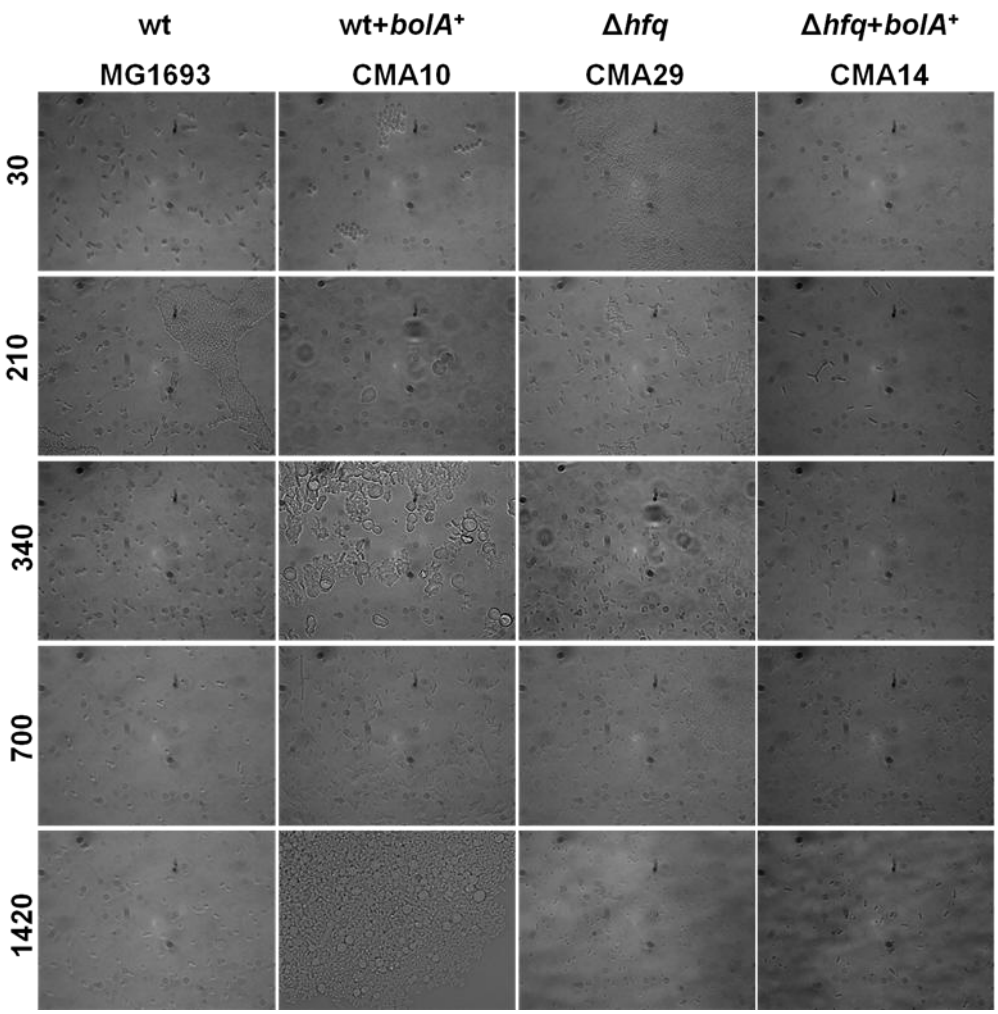


Figure 4A. Representative Differential Interference Contrast microscopy photographs overlaid in Photoshop to increase amount of data presented. Micrographs took by a DMRA microscope (Leica) at time points 30, 210, 340, and 700 and 1420 min of the growth curves of MG1693 (wt) and CMA29 (MG1693Δ*hfq*) and the respective pMAK580 transformed strains, CMA10 and CMA14.

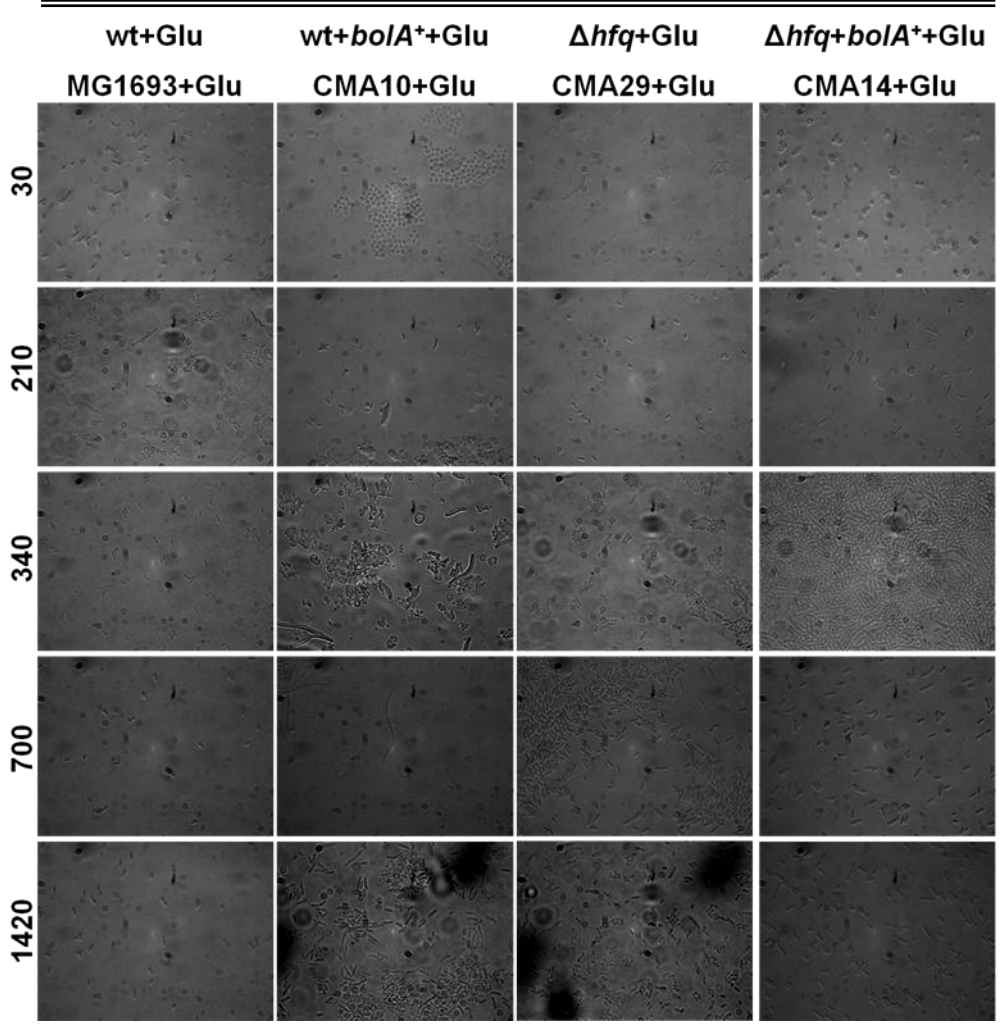


Figure 4B. Representative Differential Interference Contrast microscopy photographs. Micrographs took at the same time points of the same strains but for the glucose supplemented medium growth curves.

The combined hfq deletion and BolA overexpression morphology phenotype is privileged in minimal media and glucose depleted conditions

In glucose supplemented M9 media exponential phase, the wild type strain is characterized by small rods most of all dividing, and as numerous small rods or round spheres after one hour starving (without glucose), and again dividing 15 minutes after reversion by readdition of glucose to the depleted medium. CMA29

cells, in turn, show rod morphology with approximately twice the length of the wild type ones, also mostly dividing; after one hour starvation the cells remain long but increasing in width/thickness, a few still dividing; and upon 15 minutes of induced reversion the cells maintain their length becoming fatter, some of them round and less opaque, without apparent division processes occurring. In the later case, the absence of this regulatory protein seems to impair the growth rate preventing division, leading to the longer cells and in fewer numbers. Moreover, the absence of Hfq alone could induce cells to split into mini-cells and (rarely) Y-like after 1 hour of starvation had been imposed (Fig. 5). Unlike for LB medium, pMAK580 could induce shape disturbances both in the *hfq* deletant mutant and the wild type strain. Reversion from nutritional stress did not lead to morphological changes after 15 minutes of glucose readdition (the condition used to assess the variation in mRNA expression). That may be due to the fact that the time cells take to adapt mRNA levels does not correspond to the time they require to change both protein levels and their physiological effects, namely cell shape.

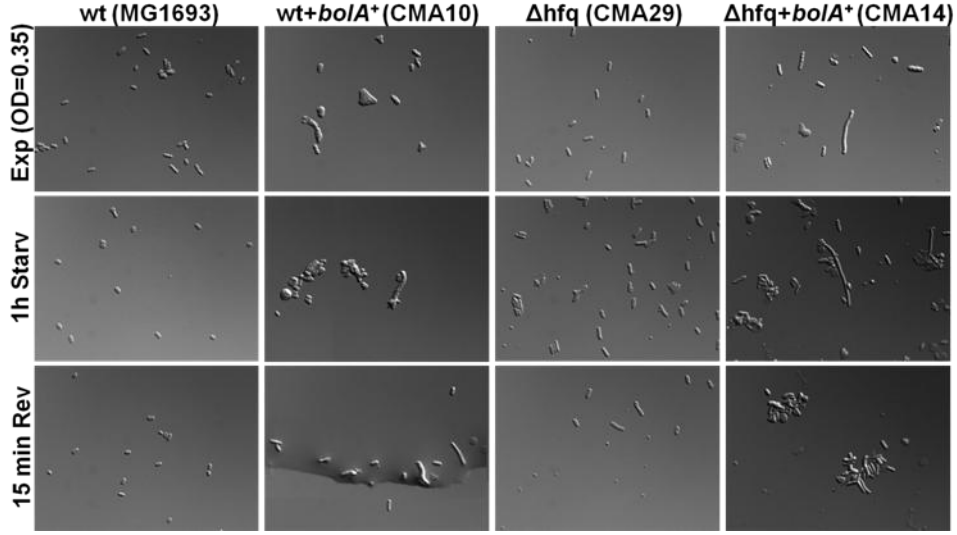


Figure 5. Representative DIC micrographs for MG1693 (wt), CMA10 (wt+*bolA*$^+$), CMA29 (Δ*hfq*) and CMA14 (Δ*hfq*+ *bolA*$^+$) transformed strains grown in M9 supplemented with glucose until OD620nm=0,35 (Exp), starved for 1 hour (1h Starv) and glucose readded for 15 minutes (15min Rev).

Hfq deletion reduces the levels of bolA mRNA and affects its stability

We wanted to evaluate if Hfq could have any effect on *bolA* mRNA levels and stability of the transcripts. Northern Blot analysis was performed to check the levels of *bolA* mRNA and the decay of this transcript in the *hfq* deletant strain, and the results were compared with the wild type. RNA was extracted from steady-state (time 0 minutes for each condition) levels and mRNA stability evaluated after extraction of RNA at times 2, 4, 8, 16, and 30 minutes after addition of rifampicin, an antibiotic that blocks *de novo* mRNA synthesis.

Both the *bolA* transcript's steady state levels and stabilities were shown to greatly decrease in CMA29 *hfq* deletion strain, when compared with the wild type MG1693, at exponential phase in minimal M9 growth medium supplemented with glucose (Fig. 6A); after one hour sugar deprivation induced at 0,35 exponential OD_{620nm} (Fig. 6B); and after 15 minutes of reversion, through the re-addition of glucose into starved medium (Fig. 6C). The *bolA* transcripts increase upon several stresses induction and rapidly decrease after the cells are relieved from stress (Santos et al., 1999) (Fig. 6D). Even though *bolA* mRNA levels are severely reduced in the *hfq* deletion strain, it's nevertheless possible to ascertain that the amounts as well as stabilities are higher in the imposed starvation condition (Fig. 6D). In both strains transcripts levels are extremely reduced upon glucose re-addition, as expected, due to the relief of carbon deprivation stress. Given that σ^S protein levels are controlled by Hfq, one cannot exclude that this is the mechanism of *bolA* mRNA transcription control. Hfq both acts at the transcription levels as it does in the half-life of *bolA* mRNA, severely reflecting on the cellular levels of this transcript.

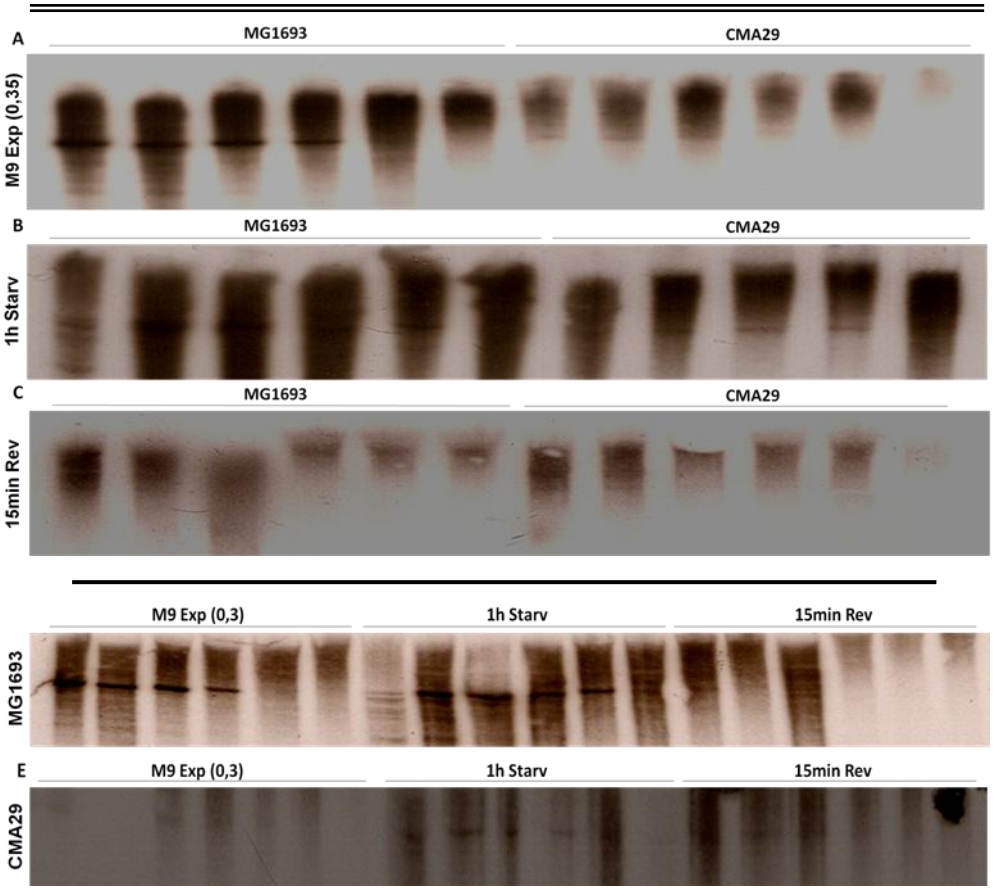


Figure 6. Northern Blot analysis of the expression of *bolA* mRNA and decay in MG1693 and CMA29 grown in M9, with total RNA samples taken at 0, 2, 4, 8, 16, and 30 minutes after addition of rifampicin. A. Exponential phase (OD_{620nm}=0.35); B. After 1h starvation; C. After 15 min of nutritional reversion; D. MG1693 *bolA* decay in the same conditions. E. CMA29 *bolA* decay in the same exponential, starvation and reversion conditions.

CONCLUSION

The levels of *bolA* transcript are affected by the presence of the RNA chaperone Hfq. The relevance of Hfq for BolA can be distinguished in the growth curves or morphologies in the presence of the pMAK580 *bolA* overexpressing plasmid. BolA is becoming increasingly relevant as more targets for its transcription factor function are known, and better understanding of physiological development and changes that it induces are achieved. Here we could prove that *hfq* deletion significantly reduces the levels of *bolA* mRNA and affect its stability.

REFERENCES

Aiba, H. 2007. Mechanism of RNA silencing by Hfq-binding small RNAs. Curr Opin Microbiol 10:134-9.

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 170:5169-76.

Ansong, C., H. Yoon, S. Porwollik, H. Mottaz-Brewer, B.O. Petritis, N. Jaitly, J.N. Adkins, M. McClelland, F. Heffron, and R.D. Smith. 2009. Global systems-level analysis of Hfq and SmpB deletion mutants in Salmonella: implications for virulence and global protein translation. PLoS One 4:e4809.

Arluison, V., S. Hohng, R. Roy, O. Pellegrini, P. Regnier, and T. Ha. 2007. Spectroscopic observation of RNA chaperone activities of Hfq in post-transcriptional regulation by a small non-coding RNA. Nucleic Acids Res 35:999-1006.

Arluison, V., C. Mura, M.R. Guzman, J. Liquier, O. Pellegrini, M. Gingery, P. Regnier, and S. Marco. 2006. Three-dimensional structures of fibrillar Sm proteins: Hfq and other Sm-like proteins. J Mol Biol 356:86-96.

Babitzke, P., and T. Romeo. 2007. CsrB sRNA family: sequestration of RNA-binding regulatory proteins. Current Opinion in Microbiology 10:156-163.

Baker, C.S., L.A. Eory, H. Yakhnin, J. Mercante, T. Romeo, and P. Babitzke. 2007. CsrA Inhibits Translation Initiation of Escherichia coli hfq by Binding to a Single Site Overlapping the Shine-Dalgarno Sequence. J Bacteriol 189:5472-81.

Bearson, S., B. Bearson, and J.W. Foster. 1997. Acid stress responses in enterobacteria. FEMS Microbiol Lett 147:173-80.

Brennan, R.G., and T.M. Link. 2007. Hfq structure, function and ligand binding. Curr Opin Microbiol 10:125-33.

Brown, L., and T. Elliott. 1996. Efficient translation of the RpoS sigma factor in Salmonella typhimurium requires host factor I, an RNA-binding protein encoded by the hfq gene. J Bacteriol 178:3763-70.

Bruckner, R., and F. Titgemeyer. 2002. Carbon catabolite repression in bacteria: choice of the carbon source and autoregulatory limitation of sugar utilization. FEMS Microbiol Lett 209:141-8.

Cao, G.J., and N. Sarkar. 1997. Stationary phase-specific mRNAs in Escherichia coli are polyadenylated. Biochem Biophys Res Commun 239:46-50.

Cunning, C., L. Brown, and T. Elliott. 1998. Promoter substitution and deletion analysis of upstream region required for rpoS translational regulation. J Bacteriol 180:4564-70.

Ding, Y., B.M. Davis, and M.K. Waldor. 2004. Hfq is essential for Vibrio cholerae virulence and downregulates sigma expression. Mol Microbiol 53:345-54.

Folichon, M., V. Arluison, O. Pellegrini, E. Huntzinger, P. Regnier, and E. Hajnsdorf. 2003. The poly(A) binding protein Hfq protects RNA from RNase E and exoribonucleolytic degradation. Nucleic Acids Res 31:7302-10.

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 88:341-6.

Geissmann, T.A., and D. Touati. 2004. Hfq, a new chaperoning role: binding to messenger RNA determines access for small RNA regulator. Embo J 23:396-405.

Guisbert, E., V.A. Rhodius, N. Ahuja, E. Witkin, and C.A. Gross. 2007. Hfq modulates the sigmaE-mediated envelope stress response and the sigma32-mediated cytoplasmic stress response in Escherichia coli. J Bacteriol 189:1963-73.

Horst W. Doelle, K.N.E.a.N.W.H. 1982. Regulation of glucose metabolism in bacterial systems. Microbial Reactions

Advances in Biochemical Engineering/Biotechnology 23:1-35.

Kawamoto, H., T. Morita, A. Shimizu, T. Inada, and H. Aiba. 2005. Implication of membrane localization of target mRNA in the action of a small RNA: mechanism of post-transcriptional regulation of glucose transporter in Escherichia coli. Genes Dev 19:328-38.

Le Derout, J., M. Folichon, F. Briani, G. Deho, P. Regnier, and E. Hajnsdorf. 2003. Hfq affects the length and the frequency of short oligo(A) tails at the 3' end of Escherichia coli rpsO mRNAs. Nucleic Acids Res 31:4017-23.

Lucchetti-Miganeh, C., E. Burrowes, C. Baysse, and G. Ermel. 2008. The post-transcriptional regulator CsrA plays a central role in the adaptation of

bacterial pathogens to different stages of infection in animal hosts. Microbiology 154:16-29.

Maki, K., K. Uno, T. Morita, and H. Aiba. 2008. RNA, but not protein partners, is directly responsible for translational silencing by a bacterial Hfq-binding small RNA. Proc Natl Acad Sci U S A 105:10332-7.

McNealy, T.L., V. Forsbach-Birk, C. Shi, and R. Marre. 2005. The Hfq homolog in Legionella pneumophila demonstrates regulation by LetA and RpoS and interacts with the global regulator CsrA. J Bacteriol 187:1527-32.

Mikulecky, P.J., M.K. Kaw, C.C. Brescia, J.C. Takach, D.D. Sledjeski, and A.L. Feig. 2004. Escherichia coli Hfq has distinct interaction surfaces for DsrA, rpoS and poly(A) RNAs. Nat Struct Mol Biol 11:1206-14.

Miller, J.H. 1972. Experiments in Molecular Genetics Cold

Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Mohanty, B.K., V.F. Maples, and S.R. Kushner. 2004. The Sm-like protein Hfq regulates polyadenylation dependent mRNA decay in Escherichia coli. Mol Microbiol 54:905-20.

Moll, I., D. Leitsch, T. Steinhauser, and U. Blasi. 2003a. RNA chaperone activity of the Sm-like Hfq protein. EMBO Rep 4:284-9.

Moll, I., T. Afonyushkin, O. Vytvytska, V.R. Kaberdin, and U. Blasi. 2003b. Coincident Hfq binding and RNase E cleavage sites on mRNA and small regulatory RNAs. Rna 9:1308-14.

Morita, T., K. Maki, M. Yagi, and H. Aiba. 2008. Analyses of mRNA destabilization and translational inhibition mediated by Hfq-binding small RNAs. Methods Enzymol 447:359-78.

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 178:1607-13.

Pirt, S.J. 1967. A kinetic study of the mode of growth of surface colonies of bacteria and fungi. J Gen Microbiol 47:181-97.

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.

Santos, J.M. 2000. Function and expression control of the morphogene *bolA*: adaptation and survival strategies of *Escherichi coli*:249.

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 32:789-98.

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 157:31-8.

Sharma, A.K., and S.M. Payne. 2006. Induction of expression of hfq by DksA is essential for Shigella flexneri virulence. Mol Microbiol 62:469-79.

Sittka, A., V. Pfeiffer, K. Tedin, and J. Vogel. 2007. The RNA chaperone Hfq is essential for the virulence of Salmonella typhimurium. Mol Microbiol 63:193-217.

Sonnleitner, E., S. Hagens, F. Rosenau, S. Wilhelm, A. Habel, K.E. Jager, and U. Blasi. 2003. Reduced virulence of a hfq mutant of Pseudomonas aeruginosa O1. Microb Pathog 35:217-28.

Takada, A., M. Wachi, and K. Nagai. 1999. Negative regulatory role of the Escherichia coli hfq gene in cell division. Biochem Biophys Res Commun 266:579-83.

Takada, A., M. Wachi, A. Kaidow, M. Takamura, and K. Nagai. 1997. DNA binding properties of the hfq gene product of Escherichia coli. Biochem Biophys Res Commun 236:576-9.

Tsui, H.C., H.C. Leung, and M.E. Winkler. 1994. Characterization of broadly pleiotropic phenotypes caused by an hfq insertion mutation in Escherichia coli K-12. Mol Microbiol 13:35-49.

Tsui, H.C., G. Feng, and M.E. Winkler. 1997. Negative regulation of mutS and mutH repair gene expression by the Hfq and RpoS global regulators of Escherichia coli K-12. J Bacteriol 179:7476-87.

Udekwu, K.I., F. Darfeuille, J. Vogel, J. Reimegard, E. Holmqvist, and E.G. Wagner. 2005. Hfq-dependent regulation of OmpA synthesis is mediated by an antisense RNA. Genes Dev 19:2355-66.

Vanderpool, C.K. 2007. Physiological consequences of small RNA-mediated regulation of glucose-phosphate stress. Curr Opin Microbiol 10:146-51.

Vanderpool, C.K., and S. Gottesman. 2004. Involvement of a novel transcriptional activator and small RNA in post-transcriptional regulation of the glucose phosphoenolpyruvate phosphotransferase system. Mol Microbiol 54:1076-89.

Wadler, C.S., and C.K. Vanderpool. 2009. Characterization of homologs of the small RNA SgrS reveals diversity in function. Nucleic Acids Res 37:5477-85.

Wilusz, C.J., and J. Wilusz. 2005. Eukaryotic Lsm proteins: lessons from bacteria. Nat Struct Mol Biol 12:1031-6.

Ziolkowska, K., P. Derreumaux, M. Folichon, O. Pellegrini, P. Regnier, I.V. Boni, and E. Hajnsdorf. 2006. Hfq variant with altered RNA binding functions. Nucleic Acids Res 34:709-20.

Appendix II

Studying BolA in pathogenic species: Burkholderia cepacia complex

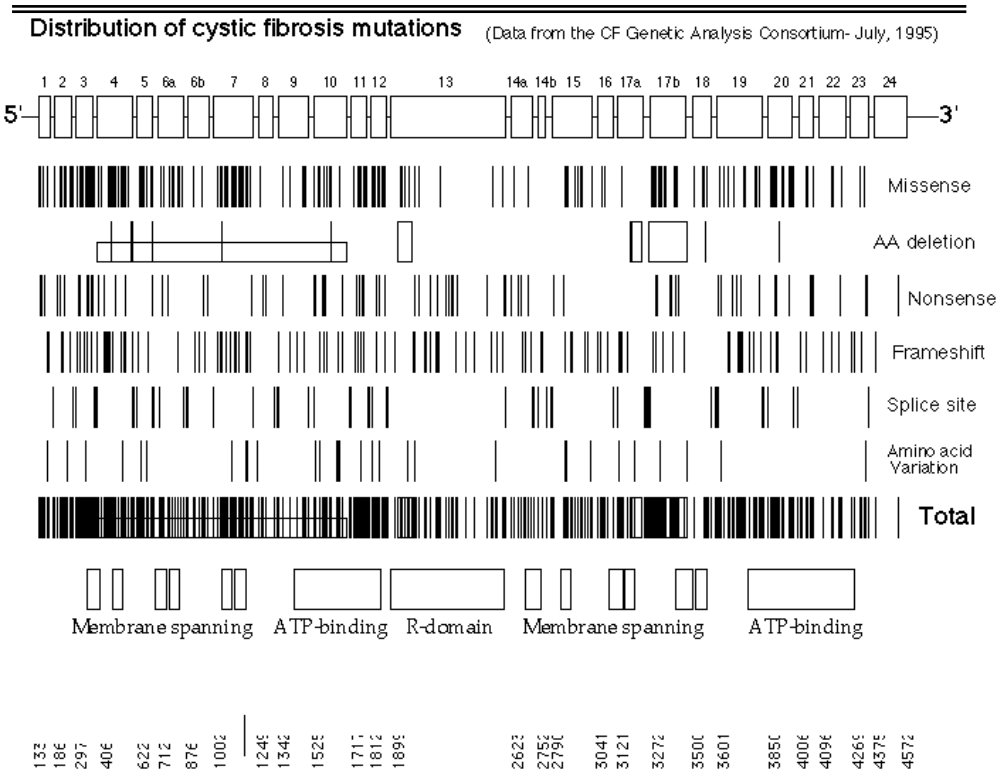
This chapter was based on Sousa, S., "*Burkholderia cepacia* complex infections in Cystic Fibrosis patients: biosynthesis and role of the exopolysaccharide and investigation of other potential virulence factors", Tese para obtenção do grau de doutor em Biotecnologia, Dec 2006.

INDEX

INTRODUCTION

The Burkholderia cepacia complex (Bcc) consists of a group of Gram-negative β-proteobacteria, which can be isolated from a variety of environmental niches from water and soils, to animals, namely humans. It was initially described to be a phytopatogen, but emerged in the 1980s to an important opportunistic human pathogen for patients with chronic granulomatous disease, in immunocompromised patients, and predominantly among cystic fibrosis (CF) patients (Mahenthiralingam et al., 2005). This group arises from commonalities of phenotypes of at least 9 different species, all capable of colonizing the lungs of CF's patients although with very different representativities (Agodi et al., 2001; Bevivino et al., 2002; Coenye et al., 2001; Cunha et al., 2003; Cunha et al., 2007; De Soyza et al., 2001; De Soyza et al., 2004; Drevinek et al., 2003; Drevinek et al., 2005; Kalish et al., 2006; Kidd et al., 2003; Mahenthiralingam et al., 2001; Moore et al., 2002; Vandamme et al., 2003; Vandamme et al., 1997; Whiteford et al., 1995). Although *B. cenocepacia* are considered of higher clinical risk in terms of death, all the others are capable of causing severe infections to this patients (Biddick et al., 2003; LiPuma et al., 1990). There is an increasing concern in Bcc strains due to their ability to survive not only in nutritionally limited environments, but even more, based on the decomposition of the antibiotics as sole carbon source, or toxics in general (Beckman and Lessie, 1979; Coenye and Vandamme, 2003).

Cystic fibrosis is the most common autosomal recessive disorder in caucasian populations, with different frequencies depending on their country origin and particular mutation analyzed (FitzSimmons, 1993). It is caused by a large number (over 1500) of mutations (Fig.1 from (Zielenski and Tsui, 1995)) in the *cftr* (Cystic Fibrosis Transmembrane Conductance Regulator) gene, 250kb (1480 aa) encoded at the chromosome 7, creating a complete lack of synthesis or defective function. This ATP-binding cassette family transporter is a cAMP-sensitive low conductance chloride channel that locates in the apical surface of epithelial cells



(Collins, 1992) but additionally seems to be involved in several signaling pathways and regulation of other transporters (Vankeerberghen et al., 2002).

Thus any mutation corrupting its function leads to simultaneous defective sodium, chloride and bicarbonate ions' stasis, inducing pathology in several organs. Namely, it has been associated with chronic pulmonary disease, pancreatic exocrine insufficiency, male infertility, and abnormally high chloride concentrations in sweat (Ratjen and Doring, 2003).

The most remarkable alterations induced by this disease occur in the patients' lungs, in an age-dependent manner. Those become progressively colonized by opportunistic, usually non-pathogenic, bacterial species. *B.cenocepacia* promotes severe decline in lung function and increased mortality, namely due to the development of cepacia syndrome with high fevers, bacteremia, severe necrotizing pneumonia and death (Govan and Deretic, 1996; Lyczak et al., 2002). The latter infections give a poor prognosis since they are very difficult to treat due

to intrinsic resistance of this microorganism to broad spectrum antibiotics (Aaron et al., 2000; Lang et al., 2000) and bactericidal components of the immune system (Baird et al., 1999). Even aggressive triple therapies rarely result in significant cfu reduction in patients (Manno et al., 2003).

Exopolysaccharides (EPS) produced by bacteria favor virulence through host defense mechanisms evasion like phagocytosis killing, scavenging the hypochlorite produced by activated phagocytes, and suppressing neutrophil and lymphocyte antibacterial functions (Pier et al., 2001). Additionally, conversion form non-mucoid to mucoid phenotype favor bacterial adhesion and biofilm development, increasing antibacterial agents resistance, thus promoting a more persistent state leading to chronic infection (Lyczak et al., 2002).

Mouse models of CF (from the Virtual Repository of Cystic Fibrosis European Network) do not reproduce human pulmonary and pancreatic insufficiencies or non-artificial persistence, sometimes providing data inconsistent with clinical history of infected patients. Those models can nevertheless produce similar to the human's disease initial phenotypes, namely developing intestinal, fertility and airway pathologies (Davidson and Rolfe, 2001).

Collaboration has been started between Control of Gene Expression Lab at ITQB and Biological Sciences Research Group at Instituto Superior Técnico (IST), after plasposon gene disruption has significantly reduced the mucoid characteristics of *Burkholderia cenocepacia* J2315 mother strain, due to inactivation of *hfq* gene. We aim to determine if the *bolA* gene of *E. coli* is able to restore the phenotype and additionally quantify and characterize differences in EPS, assay cell adhesion and biofilm development, and test for antibiotic susceptibility. Finally, if initial results are promising it would be interesting to attempt CF mouse model infection with Bcc strains deleted in *bolA*, and reversely with increased expression of BolA and evaluate for vitality deterioration, bacterial cfu, and lungs histopathology with hematoxylin-eosine.

METHODS

Bacterial strains, plasmids and genetic manipulations

A pMLBAD based shuttle vector for *Escherichia coli* and *Burkholderia cenocepacia* was used to clone *bolA* from *E. coli* (Lefebre and Valvano, 2002). The pMLBAD vector was restricted with the blunt end single cut enzyme *SmaI* (Fermentas) and cloned with different *bolA* DNA sequences amplified through *Pfu* (Fermentas) by PCR. Amplification was performed over a template of *BamHI* linearized pMAK580 with the two sets of primers: P1+X10 and X0+X10 (Freire, 2005). The transformation protocols were optimized to eliminate the false positives due to some intrinsic resistance of *E. coli* to the antibiotic used for *Burkholderia* – Trimethropim. The plasmids obtained were first screened by colony-PCR with the same primers used for amplification of the fragment of interest. This was done to check for the presence of the insert, due to the high number of false positive colonies grown. Initially 10 colonies were screened per PCR reaction, and only the ones accusing the presence of the fragment would be further analysed. That allowed a faster and less expensive evaluation. The positive colonies were then grown in liquid medium for plasmid extraction, and the plasmids were restricted with *ClaI* (NEB) to confirm the direction of the insertion present. The following table represents the sizes expected for each construction.

Table1. Cloned plasmids (see Table 2) tests: PCR amplification and digestion fragment products.

Genotype	**PCR result (DNA bp)**	***ClaI* restriction bands (bp)**
DH5αpMLBAD-Bcc	Absent	743+6032
DH5αpMLBAD/P1-X10	673	743+1965+4740
DH5αpMLBAD/X10-P1	673	743+1358+5347
DH5αpMLBAD/X0-X10	894	743+2186+4740
DH5αpMLBAD/X10-X0	894	743+1358+5568

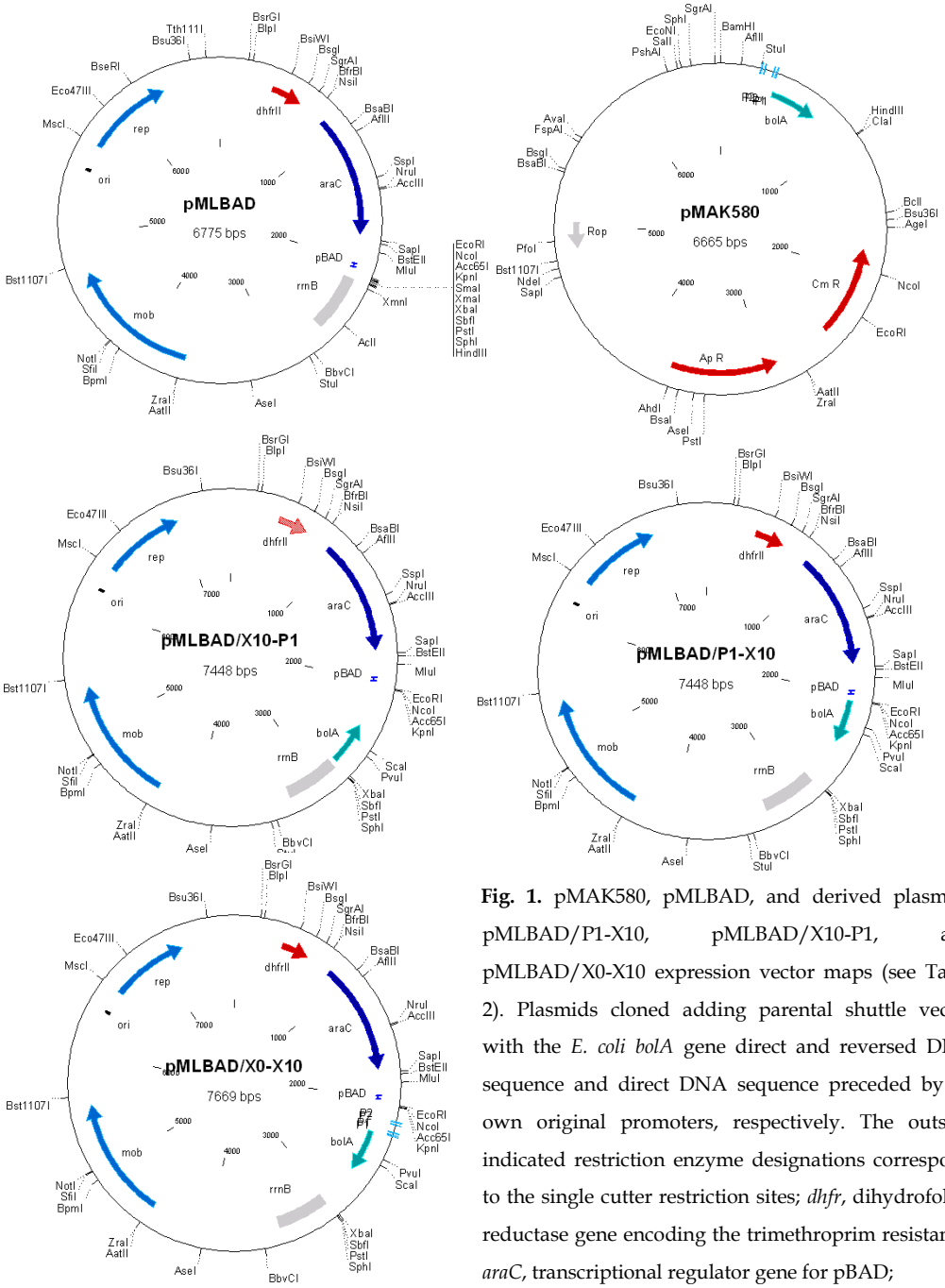


Fig. 1. pMAK580, pMLBAD, and derived plasmids pMLBAD/P1-X10, pMLBAD/X10-P1, and pMLBAD/X0-X10 expression vector maps (see Table 2). Plasmids cloned adding parental shuttle vector with the *E. coli bolA* gene direct and reversed DNA sequence and direct DNA sequence preceded by its own original promoters, respectively. The outside indicated restriction enzyme designations correspond to the single cutter restriction sites; *dhfr*, dihydrofolate reductase gene encoding the trimethroprim resistance; *araC*, transcriptional regulator gene for pBAD; pBAD, arabinose inducible promoter; *rrnB*, strong transcriptional terminator; *mob*, gene required for conjugal transfer of the plasmid; *ori*, origin of replication; *rep*, replication protein gene; *bolA*, *E. coli* morphogene cloned for study; P1, *bolA1p* promoter, mainly regulated by the σS transcription factor and induced in stationary phase and stress conditions; P2, *bolA2p* σ70-dependent and constitutive promoter.

Other primer sequences were designed for sequencing the plasmids, a forward primer pC:5'-CTCCCGCCATTCAGAGAAG-3', and a reverse one pB: 5'-CTTGGCTGTTTTGGCGGATG-3'. The sequenciation results confirmed the expected plasmids. The plasmids constructed were: pMLBAD_Bcc/X10-P1 with the inverted *bolA* sequence without promoters, designed to evaluate the effect of an eventual sRNA, responsible for controlling the respective antiparallel mRNA, if induced with arabinose; pMLBAD_Bcc/P1-X10, with the direct *bolA* sequence without promoters, and thus presenting controlled expression from the inducible arabinose promoter already present in the original pMLBAD plasmid; and pMLBAD_Bcc/X0-X10, with the direct *bolA* sequence preceded by its own promoters in order to simulate a physiologic response of this gene, if the promoter is recognized by *Burkholderia* similarly to what happens in *E. coli*.

These constructions were sent to IST to be transformed into a Δ*bolA Burkholderia cenocepacia* strain to study the effects of *bolA E. coli versus* its absence. Preliminary studies were unfruitful in the sense that what seemed to be absence of effects was confirmed to be due to the loss of plasmids previously inserted in those species. The transformation in those bacteria is difficult to confirm since the plasmids are not efficiently recovered, and on top of that plasmids are easily lost even after successful introduction in cells. New transformation and confirmation by colony-PCR (using the primers designed for clones sequencing) is currently at work.

Table 2. Plasmids used in this study

Plasmids	**Reference or source**	**Observations**
pMAK580	(Aldea et al., 1988)	plasmid overexpressing *bolA*
pMLBAD	(Lefebre and Valvano, 2002)	optimized shutle vector
pMLBAD/X10-P1	this dissertation	control vector_with reversed *bolA*
pMLBAD/P1-X10	this dissertation	*bolA* inducible by Ara vector
pMLBAD/X0-X10	this dissertation	*bolA* expressed after its own promoters vector

REFERENCES

Aaron, S.D., W. Ferris, D.A. Henry, D.P. Speert, and N.E. Macdonald. 2000. Multiple combination bactericidal antibiotic testing for patients with cystic fibrosis infected with Burkholderia cepacia. Am J Respir Crit Care Med 161:1206-12.

Agodi, A., E. Mahenthiralingam, M. Barchitta, V. Giannino, A. Sciacca, and S. Stefani. 2001. Burkholderia cepacia complex infection in Italian patients with cystic fibrosis: prevalence, epidemiology, and genomovar status. J Clin Microbiol 39:2891-6.

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 170:5169-76.

Baird, R.M., H. Brown, A.W. Smith, and M.L. Watson. 1999. Burkholderia cepacia is resistant to the antimicrobial activity of airway epithelial cells. Immunopharmacology 44:267-72.

Beckman, W., and T.G. Lessie. 1979. Response of Pseudomonas cepacia to beta-Lactam antibiotics: utilization of penicillin G as the carbon source. J Bacteriol 140:1126-8.

Bevivino, A., C. Dalmastri, S. Tabacchioni, L. Chiarini, M.L. Belli, S. Piana, A. Materazzo, P. Vandamme, and G. Manno. 2002. Burkholderia cepacia complex bacteria from clinical and environmental sources in Italy: genomovar status and distribution of traits related to virulence and transmissibility. J Clin Microbiol 40:846-51.

Biddick, R., T. Spilker, A. Martin, and J.J. LiPuma. 2003. Evidence of transmission of Burkholderia cepacia, Burkholderia multivorans and Burkholderia dolosa among persons with cystic fibrosis. FEMS Microbiol Lett 228:57-62.

Coenye, T., and P. Vandamme. 2003. Diversity and significance of Burkholderia species occupying diverse ecological niches. Environ Microbiol 5:719-29.

Coenye, T., P. Vandamme, J.R. Govan, and J.J. LiPuma. 2001. Taxonomy and identification of the Burkholderia cepacia complex. J Clin Microbiol 39:3427-36.

Collins, F.S. 1992. Cystic fibrosis: molecular biology and therapeutic implications. Science 256:774-9.

Cunha, M.V., J.H. Leitao, E. Mahenthiralingam, P. Vandamme, L. Lito, C. Barreto, M.J. Salgado, and I. Sa-Correia. 2003. Molecular analysis of Burkholderia cepacia complex isolates from a Portuguese cystic fibrosis center: a 7-year study. J Clin Microbiol 41:4113-20.

Cunha, M.V., A. Pinto-de-Oliveira, L. Meirinhos-Soares, M.J. Salgado, J. Melo-Cristino, S. Correia, C. Barreto, and I. Sa-Correia. 2007. Exceptionally high

representation of Burkholderia cepacia among B. cepacia complex isolates recovered from the major Portuguese cystic fibrosis center. J Clin Microbiol 45:1628-33.

Davidson, D.J., and M. Rolfe. 2001. Mouse models of cystic fibrosis. Trends Genet 17:S29-37.

De Soyza, A., A. McDowell, L. Archer, J.H. Dark, S.J. Elborn, E. Mahenthiralingam, K. Gould, and P.A. Corris. 2001. Burkholderia cepacia complex genomovars and pulmonary transplantation outcomes in patients with cystic fibrosis. Lancet 358:1780-1.

De Soyza, A., K. Morris, A. McDowell, C. Doherty, L. Archer, J. Perry, J.R. Govan, P.A. Corris, and K. Gould. 2004. Prevalence and clonality of Burkholderia cepacia complex genomovars in UK patients with cystic fibrosis referred for lung transplantation. Thorax 59:526-8.

Drevinek, P., O. Cinek, J. Melter, L. Langsadl, Y. Navesnakova, and V. Vavrova. 2003. Genomovar distribution of the Burkholderia cepacia complex differs significantly between Czech and Slovak patients with cystic fibrosis. J Med Microbiol 52:603-4.

Drevinek, P., S. Vosahlikova, O. Cinek, V. Vavrova, J. Bartosova, P. Pohunek, and E. Mahenthiralingam. 2005. Widespread clone of Burkholderia cenocepacia in cystic fibrosis patients in the Czech Republic. J Med Microbiol 54:655-9.

FitzSimmons, S.C. 1993. The changing epidemiology of cystic fibrosis. J Pediatr 122:1-9.

Freire, P., (ed.) 2005. Characterization of the gene *bolA* and its function in cell morphology and survival.

Govan, J.R., and V. Deretic. 1996. Microbial pathogenesis in cystic fibrosis: mucoid Pseudomonas aeruginosa and Burkholderia cepacia. Microbiol Rev 60:539-74.

Kalish, L.A., D.A. Waltz, M. Dovey, G. Potter-Bynoe, A.J. McAdam, J.J. Lipuma, C. Gerard, and D. Goldmann. 2006. Impact of Burkholderia dolosa on lung function and survival in cystic fibrosis. Am J Respir Crit Care Med 173:421-5.

Kidd, T.J., S.C. Bell, and C. Coulter. 2003. Genomovar diversity amongst Burkholderia cepacia complex isolates from an Australian adult cystic fibrosis unit. Eur J Clin Microbiol Infect Dis 22:434-7.

Lang, B.J., S.D. Aaron, W. Ferris, P.C. Hebert, and N.E. MacDonald. 2000. Multiple combination bactericidal antibiotic testing for patients with cystic fibrosis infected with multiresistant strains of Pseudomonas aeruginosa. Am J Respir Crit Care Med 162:2241-5.

Lefebre, M.D., and M.A. Valvano. 2002. Construction and evaluation of plasmid vectors optimized for constitutive and regulated gene expression in

Burkholderia cepacia complex isolates. Appl Environ Microbiol 68:5956-64.

LiPuma, J.J., S.E. Dasen, D.W. Nielson, R.C. Stern, and T.L. Stull. 1990. Person-to-person transmission of Pseudomonas cepacia between patients with cystic fibrosis. Lancet 336:1094-6.

Lyczak, J.B., C.L. Cannon, and G.B. Pier. 2002. Lung infections associated with cystic fibrosis. Clin Microbiol Rev 15:194-222.

Mahenthiralingam, E., T.A. Urban, and J.B. Goldberg. 2005. The multifarious, multireplicon Burkholderia cepacia complex. Nat Rev Microbiol 3:144-56.

Mahenthiralingam, E., P. Vandamme, M.E. Campbell, D.A. Henry, A.M. Gravelle, L.T. Wong, A.G. Davidson, P.G. Wilcox, B. Nakielna, and D.P. Speert. 2001. Infection with Burkholderia cepacia complex genomovars in patients with cystic fibrosis: virulent transmissible strains of genomovar III can replace Burkholderia multivorans. Clin Infect Dis 33:1469-75.

Manno, G., E. Ugolotti, M.L. Belli, M.L. Fenu, L. Romano, and M. Cruciani. 2003. Use of the E test to assess synergy of antibiotic combinations against isolates of Burkholderia cepacia-complex from patients with cystic fibrosis. Eur J Clin Microbiol Infect Dis 22:28-34.

Moore, J.E., B. McIlhatton, J. Buchanan, D. Gilpin, A. Shaw, V. Hall, P.G. Murphy, and J.S. Elborn. 2002. Occurrence of Burkholderia cepacia in the hospital environment. Ir J Med Sci 171:131-3.

Pier, G.B., F. Coleman, M. Grout, M. Franklin, and D.E. Ohman. 2001. Role of alginate O acetylation in resistance of mucoid Pseudomonas aeruginosa to opsonic phagocytosis. Infect Immun 69:1895-901.

Ratjen, F., and G. Doring. 2003. Cystic fibrosis. Lancet 361:681-9.

Vandamme, P., B. Holmes, T. Coenye, J. Goris, E. Mahenthiralingam, J.J. LiPuma, and J.R. Govan. 2003. Burkholderia cenocepacia sp. nov.--a new twist to an old story. Res Microbiol 154:91-6.

Vandamme, P., B. Holmes, M. Vancanneyt, T. Coenye, B. Hoste, R. Coopman, H. Revets, S. Lauwers, M. Gillis, K. Kersters, and J.R. Govan. 1997. Occurrence of multiple genomovars of Burkholderia cepacia in cystic fibrosis patients and proposal of Burkholderia multivorans sp. nov. Int J Syst Bacteriol 47:1188-200.

Vankeerberghen, A., H. Cuppens, and J.J. Cassiman. 2002. The cystic fibrosis transmembrane conductance regulator: an intriguing protein with pleiotropic functions. J Cyst Fibros 1:13-29.

Whiteford, M.L., J.D. Wilkinson, J.H. McColl, F.M. Conlon, J.R. Michie, T.J. Evans, and J.Y. Paton. 1995. Outcome of Burkholderia (Pseudomonas) cepacia colonisation in children with cystic fibrosis following a hospital outbreak. Thorax 50:1194-8.

Zielenski, J., and L.C. Tsui. 1995. Cystic fibrosis: genotypic and phenotypic variations. Annu Rev Genet 29:777-807.