The quorum sensing system and the stationary phase RpoS sigma factor of the onion pathogen *Burkholderia cepacia* Gv 1 type strain, ATCC 25416

Thesis

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The quorum sensing system and the stationary phase RpoS sigma factor of the onion pathogen *Burkholderia cepacia* Gv I type strain, ATCC 25416

Claudio Andrés Aguilar Peña

International Centre for Genetic Engineering and Biotechnology
Trieste, Italy

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Life Sciences

Director of studies: Vittorio Venturi, Ph.D.

Second supervisor: Francisco Baralle, Ph.D.

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To my parents

A mis padres

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ABSTRACT

Bacterial strains belonging to *Burkholderia cepacia* can be human opportunistic pathogens, plant growth promoting and have remarkable catabolic activity. Recently, *B. cepacia* has been thus far subdivided into several Genomovars comprising what is now known as the *B. cepacia* complex. In this thesis, the quorum sensing system of a rot onion Genomovar I type strain, ATCC 25416 is described. Quorum sensing is a cell-density dependent regulatory response, which involves the production of *N*-acyl homoserine lactones signal molecules (HSLs). The *cep* locus of *B. cepacia* ATCC 25416 coding for LuxI family CepI and LuxR family CepR proteins has been identified and characterised. The two genes have been inactivated in the chromosome and shown that CepI is responsible for the biosynthesis of a C_6-HSL and a C_8-HSL and that the *cep* locus regulates protease production as well as onion pathogenicity. A *cep-lacZ* based sensor plasmid has been constructed and used to demonstrate that CepR was specific for C_8-HSL and not for C_6-HSL, that a *cepR* knock out mutant synthesised 70 % less HSLs and that CepR had a higher specificity towards long chain HSLs. With the aid of this sensor plasmid, a novel technique aimed to identify quorum sensing-controlled (QSC) genes in *B. cepacia* is described.

In addition, the cloning and characterization of the stationary phase sigma factor gene *rpoS* of *B. cepacia* ATCC 25416 is also described. This RpoS was found to be 74 % identical to the RpoS of *Ralstonia solanacearum* but rather distant from other RpoS of gram-negative γ-*Proteobacteria*. It was established that quorum sensing in *B. cepacia* has a negative effect on *rpoS* expression as determined using an *rpoS-lacZ* transcriptional fusion; on the other hand, *rpoS*-null mutants displayed no difference in the accumulation of HSL signal molecules.

ABBREVIATIONS LIST

ACP: acyl carrier protein

ATCC: american type culture collection

BLAST: basic local alignment search tool

bp: base pair

CF: cystic fibrosis

Da: Dalton

DNA: deoxyribonucleic acid

dNTP: deoxynucleotide triphosphate

DTT: 1,4-dithio-DL-threitol

IPTG: isopropyl-β-D-thiogalactopyranoside

LB: Luria Bertani

mRNA: messenger ribonucleic acid

NADH:

OD: optical density

ORF: open reading frame

PBS: phosphate buffer saline

PCR: polymerase chain reaction

PGPR: plant growth promoting rhizobacteria

PVDF: polyvinylidene difluoride

RNA: ribonucleic acid

rRNA: ribosomal ribonucleic acid

SD: Shine-Dalgarno

TLC: thin layer chromatography

WT: wild type

X-Gal: 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside.

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INTRODUCTION

1. INTRODUCTION.

Bacteria were for a long time believed to exist as individual cells, the finding of nutrients and multiplication being their principal objectives. The discovery of intercellular communication among bacteria has led to the realisation that they are capable of coordinated activity, once erroneously believed to be restricted to multicellular organisms. The term "quorum sensing" (Fuqua *et al.*, 1996) describes one particular type of intercellular communication that takes place when bacteria are present at high cell population densities. Bacteria can achieve quorum sensing thanks to the synthesis, release, detection and response to threshold concentrations of signal molecules, originally called autoinducers. These molecules can be of different chemical nature and can be found among both Gram-positive and Gram-negative group of bacteria.

High cell densities also lead to a reduction or cessation of growth, resulting in a major switch in gene expression brought about by the alternative stationary phase sigma factor RpoS (also known as σ^{38} and σ^{s}) that allows the cells to cope with the new conditions. In *Escherichia coli*, σ^{s} regulates more than 100 genes (the σ^{s} regulon) involved in cell survival, cross protection against various stresses and in virulence (Ishihama, 2000). σ^{s} has also recently been described in the pseudomonads and shown to be a general stress regulator in *Pseudomonas putida* and *P. aeruginosa* (Ramos-Gonzalez & Molin, 1998; Suh *et al.*, 1999). In *P. fluorescens*, σ^{s} has been implicated in the production of antibiotics and in biological control by suppressing soilborne plant pathogens

(Sarniguet *et al.*, 1995). In Gram-negative bacteria, high cell densities therefore employ at least two global regulatory responses, namely quorum sensing and the switch in gene expression brought about by the stationary phase sigma factor.

This introduction will give a general overview of quorum sensing in Gram-negative bacteria, focusing on the molecular mechanisms of the genetic elements that compose the system. It will cover some aspects of the phenotypes regulated by quorum sensing and their relation to virulence. It will also introduce the role played by RpoS, described in this thesis for the first time in *B. cepacia* (Chapter 4), with respect to stationary phase gene regulation and its interaction with the quorum sensing system. Finally, current knowledge of quorum sensing in *Burkholderia cepacia* will be discussed.

1.1 Quorum sensing in Gram-negative bacteria: the model system of *Vibrio fischeri.*

The most intensely studied quorum sensing system in Gram-negative bacteria is that of the bioluminescent marine bacterium *Vibrio fischeri,* which forms a symbiotic relationship with some marine fish and squids. The best example of such a symbiosis is in *Euprymna scolopes*, where *V. fischeri* can reach extremely high densities (10^{10-11} cfu/ml) in the light organ of this squid. The bioluminescent phenotype is exploited by *Eu. scolopes* in order to perform a behavioural phenomenon called counterillumination, in which the squid camouflages itself from predators residing below, thus eliminating a visible shadow created by moonlight. *Eu. scolopes* provides, in return, nutrients to the population of *V. fischeri.* (Ruby, 1996).

Bioluminescence in this bacterium is controlled by the quorum sensing system, which is composed of two regulatory genes, *luxI* and *luxR,* coding for LuxI and LuxR, respectively. LuxI is the autoinducer synthase responsible for the production of the signal molecule *N*- (3-oxohexanoyl)-homoserine lactone (3-oxo C_6-HSL, Figure 1), and LuxR is the regulatory protein that binds the signal molecule and activates the transcription of the light production operon (Figure 1). This operon is composed of the *luxCDABEG* genes, where the *luxAB* genes encode the enzyme luciferase and *luxCDE* encode proteins required for biosynthesis of the aldehyde substrate, used by luciferase. LuxG encodes for a probable flavin reductase, and is followed by a transcriptional termination site, however its function its still unknown (Whitehead, *et al.*, 2001).

When a threshold concentration of the freely diffusible signal molecule C_6-3-oxo HSL is reached (approx 1-10 µg/ml), the LuxR protein binds the signal molecule and interacts with the *lux* box promoter sequence, thus activating the transcription of the downstream operon. This results in higher transcription (many thousand-fold) of *luxI* and hence the light production genes. At the same time, there is a reduction of the rate of transcription of *luxR*, dependent upon the presence of a *lux* box type element located within *luxD* (Shadel & Baldwin, 1992), as a way of compensating for this positive feedback (Miller & Bassler, 2001). A schematic representation of the *V. fischeri* quorum sensing system regulating luminescence is depicted in Figure 1.
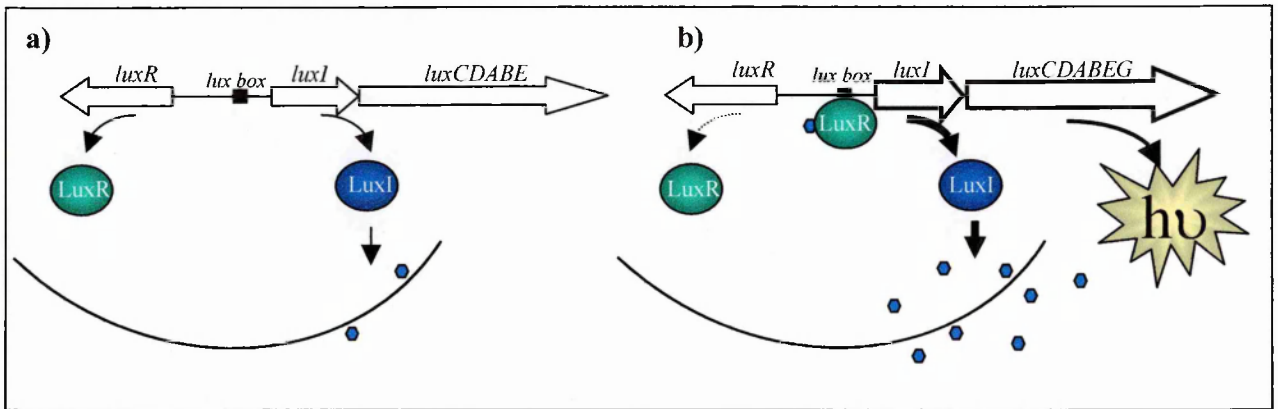
Figure 1. The *Vibrio fischeri* LuxIR quorum sensing system. a) the system is not active and there is basal transcription of *luxR*, *luxI* and *luxCDABE*. While the cell density increases, the freely diffusible signal molecule 3-oxo C_6-HSL accumulates until it reaches a threshold concentration that allows its interaction with LuxR. **b)** LuxR is bound to C_6 3-oxo-HSL, thus driving the transcription of the downstream operon while it decreases the rate of transcription of *luxR*. Picture adapted from Whitehead, *et al.* (2001).

However, the fine-tuning of the mechanism is a little more complex than mentioned above. In addition to *luxI*, there is another gene, *ainS*, involved in the modulation of the bioluminescence phenotype (Gilson *et al.*, 1995). AinS drives the synthesis of *N*-octanoyl-HSL (C_8-HSL), and this protein does not exhibit significant amino acid sequence similarity to other members of the LuxI family of proteins, indicating that AinS is a representative of a different family of autoinducer synthases (Gilson, *et al.*, 1995; Kuo *et al.*, 1994). The C_8-HSL is suggested to prevent induction at low cell densities in *V. fischeri*. The relief from this inhibition presumably occurs because of the accumulation at high population densities of sufficient amounts of 3-oxo C_6-HSL, to

outcompete C$_8$-HSL for the available LuxR, as well as from the increased availability of active LuxR in the late exponential phase of growth (Kuo *et al.*, 1996).

1.2   Biosynthesis of cell-cell communication molecules.

A general mechanism proposed for HSLs biosynthesis is depicted in Figure 2, in which a HSL synthase uses the substrates *S*-adenosyl-L-methionine (SAM) and acylated acyl carrier protein (acyl-ACP) in a proposed "bi-ter" sequentially ordered reaction (Parsek *et al.*, 1999; Val & Cronan, 1998). In this reaction, the acyl chain is presented to the HSL synthase as a thioester of the ACP phosphopantetheine prosthetic group, which results in nucleophilic attack on the 1-carbonyl carbon by the amine of SAM in the acylation reaction. Lactonization occurs by nucleophilic attack on the γ carbon of SAM by its own carboxylate oxygen to produce the HSL product. The *N*-acylation reaction, involving an enzyme-acyl-SAM intermediate, is thought to occur first because butyryl-SAM acts as both a substrate and as an inhibitor for the *P. aeruginosa* HSL synthase, RhlI, to produce C4-HSL (Parsek, *et al.*, 1999). A unique aspect of the HSL synthesis mechanism is that the substrates adopt roles that differ quite dramatically from their normal cellular functions. SAM usually acts as a methyl donor, whereas acyl-ACPs are components of the fatty acid biosynthetic pathway and had not been implicated in cell-cell communication until their discovery as acyl chain donors in HSL synthesis (Moré *et al.*, 1996).
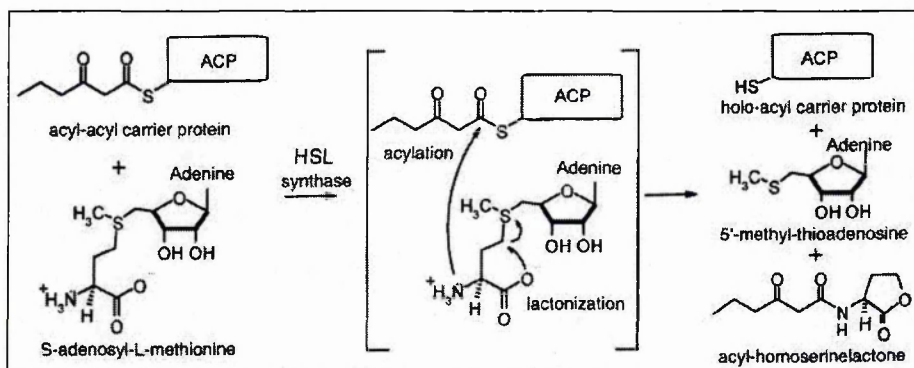
Figure 2. General features of the HSL biosynthesis reaction. Two substrates, acyl-ACP and SAM, bind to the enzyme. After the acylation and lactonization reactions, the product HSL and byproducts holo-ACP and 5'-methylthioadenosine are released. Picture extracted from Watson *et al.* (2002).

The HSL synthases belonging to the LuxI family are on average 200 amino acids in size, and in different bacterial species produce HSLs that vary from 4 to 14 carbon in acyl chain length, oxidation at the C3 position and saturation of the acyl chain (Fuqua, *et al.*, 1996; Kuo, *et al.*, 1994). This variability is a function of the enzyme acyl chain specificity and may be influenced by the available cellular pool of acyl-ACPs (Jiang *et al.*, 1998; More, *et al.*, 1996). More than 40 HSL synthases, similar to the archetype LuxI, have been characterized, and they share four motifs of conserved sequence (Fuqua & Winans, 1994). Within these motifs, there is on average 37% identity with eight residues that are absolutely conserved. When mutated, the most conserved residues impact catalysis by the LuxI and RhlI synthases (Parsek *et al.*, 1997).

Interestingly, some Gram-negative bacteria have HSL synthases that are unrelated in sequence to the LuxI family enzymes although they are proposed to catalyze an identical reaction. These include LuxM from *Vibrio harveyi* (Bassler *et al.*, 1993), HdtS

from *Pseudomonas fluorescens* (Laue *et al.*, 2000), VanM from *Vibrio anguillarum* (Milton, *et al.*, 2001) and, as mentioned above, AinS from *V. fischeri* (Gilson, *et al.*, 1995).

1.2.1 Non-HSL cell-cell communication molecules

In addition to HSL, alternative cell density dependent signalling molecules can be found among Gram-negative bacteria. Some examples are in *Ralstonia solanacearum*, a phytopathogen that produces 3-hydroxypalmitic acid methyl ester as a novel signalling molecule together with C_8-HSL (Flavier *et al.*, 1997a); *Xanthomonas campestris* pv. *campestris*, a cabbage pathogen that produces a diffusible signal factor (DSF) the structure of which seems not to be a HSL and has yet to be fully characterised (Barber *et al.*, 1997); *Pseudomonas aeruginosa*, which produces a 2-heptyl-3-hydroxy-4-quinolone together with C_4-HSL and C_{12}-3-oxo-HSL (McKnight *et al.*, 2000; Pesci *et al.*, 1999). Moreover, butyrolactones have been isolated from *Pseudomonas aureofaciens* cell-free culture supernatants (Gamard *et al.*, 1997), and a novel family of signalling compounds identified as diketopiperazines (DKPs) were isolated from cell-free culture supernatants of *Pseudomonas putida* (Degrassi *et al.*, 2002), *Pseudomonas aeruginosa, Pseudomonas fluorescens, Pseudomonas alcaligenes, Enterobacter agglomerans, Proteus mirabilis* and *Citrobacter freundi* (Holden *et al.*, 1999). Although these non-HSL molecules can modulate the activity of a number of LuxR-based quorum sensing systems, the concentrations required are much greater than those of the cognate HSL. On the other hand, some of the DKPs act antagonistically, reducing C_6-3-oxo-

HSL-mediated bioluminescence, suggesting that they may be able to compete for LuxR

binding (Holden, *et al.*, 1999).

Interestingly, AI-2 (structurally different to the acyl homoserine lactone molecule)

synthesised by *Vibrio harveyi*, has been proposed as a universal signalling molecule,

which would facilitate inter-species communication (Surette *et al.*, 1999). LuxS, the

protein that drives the synthesis of AI-2, has been showed to exist in over 30 species of

both Gram-negative and Gram-positive bacteria (Bassler, 1999; Surette, *et al.*, 1999),

thus defining a new family of proteins involved in cell-cell communication molecules

production (Surette, *et al.*, 1999). The structure of AI-2 has been recently resolved and

shown to be a furanosyl borate diester (Chen *et al.*, 2002).

1.3   The LuxR transcriptional activator class of proteins.

The LuxR-type proteins facilitate responses to HSLs through a series of recognizable

steps including:

i   specific binding of cognate HSL.

ii   conformational changes and multimerisation of the protein following binding of
       the signal molecule.

iii   binding or release of specific regulatory sequences upstream of target genes.

iv   activation of transcription.

LuxR-type proteins are on average 250 amino acids in size and alignment analysis show

that they share a sequence identity of 18-23% (Fuqua *et al.*, 2001).

Genetic analyses of LuxR-type proteins show that they are composed of two functional domains: an amino-terminal domain with an HSL binding region and a carboxy-terminal, which includes a helix-turn-helix (HTH) DNA binding domain. Mutations in key residues within the amino-terminal domain abolish or severely reduce the response to HSL (Fuqua, *et al.*, 2001; Hanzelka & Greenberg, 1995; Slock *et al.*, 1990). On the other hand, the carboxy-terminal domain, when over-expressed *in vivo*, is sufficient to activate transcription (Choi & Greenberg, 1991), and has been shown *in vitro* to facilitate RNA polymerase binding to target promoter DNA (Stevens *et al.*, 1994). A model derived from this data is one in which the inhibition of the carboxy-terminal domain by the amino-terminal region is relieved when this last region binds the HSL (Choi & Greenberg, 1991; Choi & Greenberg, 1992). Another proposed consequence of the interaction between many LuxR homologues and their cognate HSL is the promotion of multimerisation of the protein (Luo & Farrand, 1999).

LuxR of *V. fischeri* has been identified as an amphipathic membrane-associated transcription factor (Kolibachuk & Greenberg, 1993). Supporting this hypothesis, it has been demonstrated that the LuxR-type protein TraR from *A. tumefaciens*, can be found in an inactive form associated as monomers with the inner face of the cytoplasmic membrane, where it is shielded from nascent HSL (3-oxo C_8-HSL) produced by the HSL synthase TraI within the cell (More, *et al.*, 1996). When the threshold concentration of HSL is reached, it can interact with its binding site on TraR. This interaction is predicted to be at a stoichiometry of 1:1 (Zhu & Winans, 1999), and it allows TraR to dimerise and be released from the membrane into the cytoplasm, probably due to a conformational change in the protein (Qin *et al.*, 2000). Recently,

crystallographic data shows the interaction of TraR with both the cognate HSL and a target DNA sequence containing the canonical *tra* box (Vannini *et al.*, 2002; Zhang *et al.*, 2002), confirming the interaction of the C-terminal domain with the DNA duplex via the HTH motif.

As mentioned above, the interaction with the cognate HSL makes the LuxR homologues become active, thus allowing the binding to the corresponding *lux*-type box of the quorum sensing regulon. The *lux*-type boxes are elements of inverted repeated sequence, ranging from 18 to 22 bp and associated with the promoters of genes regulated by LuxR-type proteins from several different bacteria. Many *lux*-type boxes are positioned just upstream of the -35 sequences of regulated promoters, suggesting that LuxR-type proteins can interact directly with the RNA polymerase (Fuqua, *et al.*, 2001).

In *V. fischeri*, the *lux* box is 20 bp in length, centered at the –42.5 position relative to the *luxI* transcription start site (Egland & Greenberg, 1999; Egland & Greenberg, 2000). Both the structure and the positioning of the *lux* box suggest that LuxR acts as a ambidextrous activator, having thus a requirement of interaction with the αCTD of RNA polymerase as well as making contact with other regions of the protein downstream of their binding site (Egland & Greenberg, 1999; Finney *et al.*, 2002).

1.4   Different phenotypes are modulated by Quorum sensing

One single bacterium cannot evade the barriers that the colonisation of a target organism represents. Quorum sensing is the genetic tool that bacteria use to

coordinately take advantage of the number of individuals in order to establish such efficient colonisation. The benefits of a unified response may include improved access to complex nutrients or environmental niches, collective defence against other competitive micro-organisms or eukaryotic host defence mechanisms and optimisation of population survival by differentiation into morphological forms better adapted to a given environmental threat (Williams *et al.*, 2000).

As mentioned above, one of the first and best-described phenotypes to be controlled by quorum sensing is bioluminescence in the marine bacterium *V. fischeri*. Since then, the fact that expression of certain genes is correlated with high cell density has become more the rule than a curious and isolated phenomenon performed by an esoteric light-emitting marine bacterial species. In fact, the list of organisms for which a quorum sensing system has been identified is constantly growing. It is now evident that diverse Gram-negative bacteria produce HSLs, with examples of species belonging to the α, β, and γ, but not the δ or ε subdivision of the phylum *Proteobacteria*. Quorum sensing modulates a variety of physiological processes, including bioluminescence, swarming, swimming and twitching motility, antibiotic biosynthesis, biofilm differentiation and conjugation (Camara *et al.*, 2002; Williams, *et al.*, 2000). In Table 1, some example organisms are described by their quorum sensing genetic elements and the phenotypes modulated by them, where known. As can be observed, some opportunistic human pathogens such as *Pseudomonas aeruginosa*, *Aeromonas hydrophila* or *Chromobacterium violaceum* produce HSLs that are used to modulate the production of virulence determinants as exoenzymes or proteases. These are capable of contributing to virulence by causing tissue damage (Table 1). Interestingly, obligate human pathogens

such as *Haemophilus influenzae, Neisseria meningitidis*, and *Neisseria gonorrhoeae* do

not seem to make HSLs (Camara, *et al.*, 2002).

Table 1. Example of microorganisms, their respective quorum sensing system(s), and the phenotypes modulated in each case, where known.

BACTERIUM	LUX I/R HOMOLOGUES	MAJOR AHL	MODULATED PHENOTYPE	REFERENCE
Aeromonas hydrophila	AhyR, AhyI	C4-HSL	Extracellular protease, biofilm formation	(Lynch *et al.*, 2002; Swift *et al.*, 1999)
Aeromonas salmonicida	AsaR, AsaI	C4-HSL	Extracellular protease	(Swift *et al.*, 1997)
Agrobacterium tumefaciens	TraR, TraI	3-oxo-C8-HSL	Conjugation	(Fuqua & Winans, 1994; Piper *et al.*, 1999)
Chromobacterium violaceum	CviR, CviI	C6-HSL	Antibiotics, violacein, exoenzymes, cyanide	(Chernin *et al.*, 1998; McClean *et al.*, 1997)
Erwinia carotovora	CarR, ExpR CarI (or ExpI)	3-oxo-C6-HSL	Carbapenem antibiotic, exoenzymes, exopolysaccharide	(Andersson *et al.*, 2000; Bainton *et al.*, 1992; Pirhonen *et al.*, 1993; Swift *et al.*, 1993)
Escherichia coli	SdiA	Unknown	Cell division	(Sitnikov *et al.*, 1996)
Pantoea stewartii	EsaR, EsaI	3-oxo-C6-HSL	Exopolysaccharide	(Beck von Bodman & Farrand, 1995)
Pseudomonas aeruginosa	LasR, LasI	3-oxo-C12-HSL	Exoenzymes, Xcp, biofilm formation, RhlR, cell-cell spacing.	(Chapon-Herve *et al.*, 1997; Gambello & Iglewski, 1991; Glessner *et al.*, 1999; Passador *et al.*, 1993)
	RhlR, RhlI (VsmR, VsmI)	C4-HSL	Exoenzymes, cyanide, RpoS, lectins, pyocyanin, rhamnolipid..	(Latifi *et al.*, 1996; Latifi *et al.*, 1995; Pearson *et al.*, 1997; Winson *et al.*, 1995)
Pseudomonas aureofaciens	PhzR, PhzI	C6-HSL	Phenazine antibiotic	(Pierson *et al.*, 1994; Wood *et al.*, 1997)
Pseudomonas fluorescens	PhzR, PhzI HdtS	C6-HSL; C10-HSL; 3OH-7-*cis*-C14-HSL	Phenazine antibiotic	(Laue, *et al.*, 2000; Shaw *et al.*, 1997)
Ralstonia solanacearum	SolR, SolI	C8-HSL	Unknown	(Flavier *et al.*, 1997b)
Rhizobium leguminosarum	RhiR	3OH-7-*cis*-C14-HSL	Nodulation, bacteriocin, stationary phase survival	(Rodelas *et al.*, 1999; Thorne & Williams, 1999)
Rhodobacter sphaeroides	CerR, CerI	7-*cis*-C14-HSL	Community escape	(Puskas *et al.*, 1997)
Serratia liquefaciens	SwrR, SwrI	C4-HSL	Swarming, protease	(Eberl *et al.*, 1996; Givskov *et al.*, 1997; Lindum *et al.*, 1998)
Vibrio anguillarum	VanR, VanI	3-oxo-C10-HSL	Unknown	(Milton *et al.*, 1997)
	VanT	3-OH-C6-HSL; C6-HSL	EmpA expression, pigment production, and biofilm formation.	(Croxatto *et al.*, 2002)
Vibrio fischeri	LuxR, LuxI	3-oxo-C6-HSL	Bioluminescence	(Engebrecht & Silverman, 1987)

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1.5   The stationary phase sigma factor RpoS (σ^S).

Another very effective mechanism employed by bacteria to bring about such a major switch in gene expression at high cell densities, referred mainly to stationary phase of growth, is the use of the RpoS alternative sigma factor that alters RNA polymerase core specificity. The RNA polymerase holoenzyme is composed of the core enzyme, with the catalytic activity of RNA polymerisation ($\alpha_2\beta\beta'$), and one of the several different species of sigma (σ) factors present in bacteria, that bind reversibly to the core. Each σ factor plays a determining role in the specificity of transcription initiation, being released during elongation of the RNA chain (Helmann & Chamberlin, 1988).

In *Escherichia coli*, seven different species of σ subunits have been identified, each participating in transcription of a specific set of genes, these are σ^{70} (Hawley & McClure, 1983), σ^N (Hunt & Magasanik, 1985), σ^H (σ^{32}) (Grossman *et al.*, 1984), σ^F (σ^{28}) (Arnosti & Chamberlin, 1989), σ^E (σ^{24}) (Erickson & Gross, 1989), σ^{FecI} (Angerer *et al.*, 1995), and the stationary phase sigma factor σ^S (σ^{38}) (Lange & Hengge-Aronis, 1991).

The σ^S protein is encoded by the *rpoS* gene and regulates the expression of approximately 100 genes (Ishihama, 2000). It has been demonstrated that σ^S and σ^S-dependent genes are not only induced in the stationary phase, but they actually respond to different stress conditions as for example osmolarity upshift (Hengge-Aronis, 1996) or heat shock (Muffler *et al.*, 1997). Therefore, σ^S, or RpoS, is considered as the master regulator of the general stress response, which is triggered by many different stress

conditions and renders bacteria broadly resistant even to stresses that they have not yet

experienced. Thus, the major function of the general stress response is not specific

(regulating only proteins that overcome a specific stress situation), but preventative

(Hengge-Aronis, 2002a).

The regulation of σ^S is one of the most complex systems present in *E. coli*, and many

studies have so far concentrated at the post-transcriptional level and on σ^S proteolysis.

The basic control of *rpoS* translation uses *rpoS* mRNA secondary structure, the Hfq and

HU proteins, and small RNAs such as the DsrA mRNA. In addition, The increase in σ^s

levels in stationary phase occurs also in part due to a great increase in stability of the

protein mediated by the activity of the ClpXP protease, which recognizes a 20 amino

acid stretch between residues 170 and 190 and by the response regulator RssB (Hengge-

Aronis, 2002b).

1.5.1 Quorum sensing and RpoS (σ^S).

As mentioned above, the stationary phase sigma factor RpoS has been implicated in the

change in gene expression necessary for the adaptation to stationary phase (Gerard *et

al.*, 1999; Ramos-Gonzalez & Molin, 1998; Suh, *et al.*, 1999). It has also been shown to

be involved in the regulation of expression of virulence factors (Corbell & Loper, 1995;

Iriarte *et al.*, 1995; Sarniguet, *et al.*, 1995).

The fact that sometimes the high cell density in a bacterial population turns out to be the

inducing signal for the stationary phase-regulated genes is enough to think of a possible

correlation between RpoS and quorum sensing. Nevertheless, although there is some

evidence that highlights the importance of RpoS in quorum sensing and *vice-versa*, there are conflicting data regarding this reciprocal regulation. In studies conducted in *R. solanacearum*, indirect evidence show that RpoS regulates the HSL production by the negative modulation of *solR* and *solI* expression, and that *rpoS* is not regulated by the quorum sensing system (Flavier *et al.*, 1998).

On other hand, in *P. aeruginosa* quorum sensing was shown to modulate the expression of *rpoS* (Latifi, et al., 1996). However, a recent study demonstrated that is actually RpoS that regulates *rhlI*, in other words, quorum sensing (Whiteley et al., 2000). In *E. coli* there is also conflicting data about the induction of *rpoS*, but the evidence indicates that quorum sensing does not play a significant role in the regulation of this protein (Hengge-Aronis, 2002b).

1.6   *Burkholderia cepacia.*

Burkholderia cepacia, a nutritionally versatile Gram-negative organism, was first described in 1950 by W. H. Burkholder as the phytopathogen responsible for the rot of onions (Burkholder, 1950). This organism is inherently resistant to multiple antibiotics, can metabolise diverse substrates and is found in water, soil, moist environments and in the rhizosphere of crop plants (Holmes *et al.*, 1998; Parke & Gurian-Sherman, 2001). It has been shown that *B. cepacia* has remarkable potential as an agent for both biodegradation and biocontrol, thus it is also being considered as a plant growth promoting rhizobacterium (McLoughlin *et al.*, 1992). Such properties have attracted considerable interest from agricultural researchers attempting development of *B.*

cepacia strains for use as biological control agents to combat soilborne plant pathogens and to decontaminate soils containing toxic pesticides and herbicides (Govan & Vandamme, 1998). However, while beneficial in agriculture, this organism has emerged as an important opportunistic human pathogen for cystic fibrosis, hospitalized and immunocompromised patients (Holmes, *et al.*, 1998).

Formerly a member of the genus *Pseudomonas*, it was reclassified as *Burkholderia cepacia* in 1992 and it was assigned as the type species for the new genus *Burkholderia* (Yabuuchi *et al.*, 1992). In contrast to the genus *Pseudomonas*, the genus *Burkholderia* belongs to the β-subdivision of the phylum *Proteobacteria* (Coenye *et al.*, 2001c).

From the mid-1990s on, several researchers noted that there was a marked heterogeneity among *B. cepacia* strains isolated from different ecological niches. The high diversity among presumed *B. cepacia* strains and the lack of reliable identification schemes led to a polyphasic taxonomic study demonstrating that presumed *B. cepacia* strains isolated from CF patients and other sources belonged to at least five distinct genomic species or genomovars. The term genomovar refers to a group of organisms with phenotypic similarity but genotypic uniqueness (Coenye, *et al.*, 2001c). Further work has identified at least nine genomovars, which constitute the *B. cepacia* complex (Table 2).

The different genomovars of the *B. cepacia* complex are very closely related, with few if any biochemical reactions able to separate them, they share a high degree of 16S rDNA (98-100%) and *recA* (94-95%) sequence similarity and moderate levels of DNA-DNA hybridisation (30-60%) (Vandamme *et al.*, 2002).

Table 2. The *Burkholderia cepacia* complex.

Burkholderia cepacia Complex member	Species name	Reference(s)
Genomovar I	*B. cepacia*	(Palleroni & Holmes, 1981; Vandamme *et al.*, 1997)
Genomovar II	*B. multivorans*	(Vandamme, *et al.*, 1997)
Genomovar IIIa,b	*	(Balandreau *et al.*, 2001; Moore *et al.*, 2002; Vandamme, *et al.*, 1997)
Genomovar IV	*B. stabilis*	(Vandamme *et al.*, 2000)
Genomovar V	*B. vietnamiensis*	(Gillis *et al.*, 1995)
Genomovar VI	*	(Coenye *et al.*, 2001a)
Genomovar VII	*B. ambifaria*	(Coenye *et al.*, 2001b)
Genomovar VIII	*B. anthina*	(Vandamme, *et al.*, 2002)
Genomovar IX	*B. pyrrocinia*	(Vandamme, *et al.*, 2002)

*Denotes that no species name has been yet assigned to this genomovar.

Recent data show that *B. cepacia* genomovar III is the most prevalent genomovar in CF patients examined from USA, Canada and Italy, causing more than the 50% of the infections (Mahenthiralingam *et al.*, 2002). Manifestations of *B. cepacia* infections are varied and include asymptomatic carriage, a gradual decline in lung function, accompanied by necrotising pneumonia and occasionally bactereamic complications, which affects approximately 20 % of cystic fibrosis patients (Isles *et al.*, 1984).

On the other hand, it has become evident that the genome of *B. cepacia* has an unusual and characteristic organization having multiple replicons (Cheng & Lessie, 1994). As

an example, a physical map of the 8.1 Mbp genome of *B. cepacia* ATCC 25416

(belonging to genomovar I) has been constructed and consist of three chromosomes of

3.65 Mb, 3.17 Mb, 1.07 Mb and a large plasmid of 200 kbp in size (Rodley *et al.*,

1995).

B. cepacia is also notable in harbouring an extensive array of insertion sequences (IS

elements), which have been isolated because of their ability to promote genomic

rearrangements (Barsomian & Lessie, 1986; Byrne & Lessie, 1994) and to increase the

expression of neighbouring genes (Haugland *et al.*, 1990; Scordilis *et al.*, 1987). Such

elements appear to contribute significantly to the genomic plasticity of *B. cepacia* and

to play an important role in the evolution of novel catabolic functions (Daubaras *et al.*,

1995; Haugland, *et al.*, 1990).

1.6.2 Quorum sensing in *Burkholderia cepacia*.

The expression of multiple virulence factors has been linked to quorum sensing in

Gram-negative bacteria. For example, in the opportunistic *P. aeruginosa*, the RhlIR

system activates expression of the *rhlAB* rhamnolipid synthesis genes, *rhlI*, and to some

extent *lasB*, encoding an elastase (Brint & Ohman, 1995; Latifi, *et al.*, 1996; Ochsner *et

al.*, 1994; Ochsner & Reiser, 1995; Pearson, *et al.*, 1997). Other virulence factors and

secondary metabolites, including pyocyanin, cyanide, and chitinase, are positively

regulated by the *rhl* system. (Latifi, *et al.*, 1995; Ochsner & Reiser, 1995).

The quorum sensing system of *B. cepacia*, *cepIR*, has been recently identified in the

strain K56-2 (a clinical isolate from a cystic fibrosis patient), belonging to genomovar

III. Quorum sensing in this strain has been implicated in the modulation of the expression of virulence factors such as siderophores (iron transport molecules) and an extracellular protease (Lewenza *et al.*, 1999; Lewenza & Sokol, 2001). The LuxI homologue CepI directs the synthesis of C_8-HSL and C_6-HSL, while the LuxR homologue CepR is the cognate transcriptional regulator protein (Lewenza, *et al.*, 1999).

Recently, it has also been shown that the *cepIR* genes are present and conserved among the *B. cepacia* complex members (Gotschlich *et al.*, 2001). Several members of the *B. cepacia* complex were analysed for the presence of HSL molecules, for the *cepIR* homologues and for their possible involvement in the regulation of production of HSL and other extracellular activities (enzymes, siderophores). All strains tested contained the *cepIR* locus and interestingly, no direct correlation was found between quorum sensing and the extracellular activities. This is intriguing since it can be speculated that a non-HSL producer *B. cepacia* strain could be less pathogenic, however two of the major transmissible strains in CF are non-HSL producers (Mahenthiralingam *et al.*, 1996; Sajjan *et al.*, 1995). On the other hand, *B. cepacia* H111 (genomovar III) regardless of being a high level HSL producer is only a mild coloniser in cystic fibrosis patients (Gotschlich, *et al.*, 2001). Nevertheless, more data is required in order to consistently assume a correlation between *in vivo* HSL production and virulence.

It has also been shown that quorum sensing is a major checkpoint for biofilm formation in *Burkholderia cepacia* H111 (Huber *et al.*, 2001; Huber *et al.*, 2002). However, when different members of the *B. cepacia* complex were analysed, the correlation between the biofilm formation and HSL synthesis was shown to be more complex (Conway *et al.*,

2002), and it is suggested that growth conditions can be of crucial importance. In any case, HSL production likely plays a role in the ecology and pathogenesis of the *B. cepacia* complex (Conway, *et al.*, 2002).

1.7   Aim and outline of this thesis.

The experiments described in this thesis were aimed at contributing to our knowledge in the field of quorum sensing in *Burkholderia cepacia* by making use of the onion pathogen type strain ATCC 25416. The quorum sensing system of two clinical isolates of *B. cepacia*, belonging to genomovar III, have only been described recently and associated with the modulation of different phenotypes such as extracellular proteases, siderophores or biofilm formation (Huber, *et al.*, 2001; Lewenza, *et al.*, 1999). In the second chapter, the quorum sensing system of *B. cepacia* ATCC 25416, belonging to genomovar I, is identified and characterised. The system was found to be positively autoregulated, and to be more responsive to long chain HSLs than short chain, with C_8-HSL as the molecule that maximally activates the system.

The third chapter describes a molecular study of some of the phenotypes that are modulated by quorum sensing in this strain. It is shown that onion pathogenicity is diminished in the quorum sensing null-mutant, and this is correlated with the lower expression of the extracellular enzyme polygalacturonase. This chapter also describes the identification of putative quorum sensing-regulated loci in *B. cepacia*. The identification of several loci is shown and their putative regulation by quorum sensing is discussed.

The fourth chapter deals with the stationary phase sigma factor RpoS (σ^S) in *B. cepacia*. This is the first report of σ^S in the *B. cepacia* complex and only the second in the β-*Proteobacteria* phylum. It is shown that RpoS in *B. cepacia* is necessary for adaptation to heat and oxidative stress, whereas it was not important for osmotic shock adaptation. It was determined that a genetic background without RpoS has no influence on the accumulation of the HSL molecules produced by *B. cepacia*. On the other hand, *rpoS* promoter activity is positively influenced by the absence of the quorum sensing system. The interaction between quorum sensing and RpoS in *B. cepacia* is discussed.

The last chapter presents an overall discussion of the results presented throughout this thesis.

THE QUORUM SENSING SYSTEM OF THE ONION PATHOGEN

Burkholderia cepacia GV. I TYPE STRAIN ATCC 25416

2.1   INTRODUCTION.

Burkholderia cepacia was first described as a potent phytopathogen responsible for the

bacterial rot of onions (Burkholder, 1950). Bacterial strains belonging to *B. cepacia* are

recognised as a major opportunistic pathogen in patients with fibrocystic lung disease

(Govan & Deretic, 1996). *B. cepacia* strains can degrade complex herbicides and

pesticides (Daubaras et al., 1995) and also behave as plant growth promoting

rhizobacteria (PGPR) by suppressing soilborne plant pathogens (Bevivino et al., 1994;

Cartwright D.K. et al., 1995; McLoughlin et al., 1992). Recently, strains currently

identified as *B. cepacia* have been sub-divided into further groups or subpopulations

termed genomovars (Vandamme et al., 2000). The term genomovar refers to a group of

strains with phenotypic similarity but genotypic uniqueness, the group of genomovars in

this case is called the *B. cepacia* complex which comprises of at least of nine genomic

species (Coenye et al., 2001; Vandamme et al., 2002; Vandamme, et al., 2000).

Taxonomic studies have shown that the *B. cepacia* complex is a very heterogeneous

group of genotypically distinct strains that show a low level of DNA hybridization.

Quorum sensing is a mechanism for regulating gene expression in response to changes in

cell density of a bacterial population (Fuqua et al., 2001). In Gram negative bacteria, it

involves the biosynthesis of *N*-acyl homoserine lactones (HSLs) autoinducer signalling

molecules, produced by an autoinducer synthase protein belonging to the LuxI family,

and of a transcriptional regulator which is part of the LuxR family. The autoinducers

produced by different bacterial species differ in the length and structure of the acyl chain

and they are believed to be readily diffusible across the cell envelope into the growth

medium where they accumulate. Accumulation continues until the cell density, consequently also HSL concentration, reaches a quorum thereby activating the 'LuxR' type protein, which modulate the expression of target genes. Quorum sensing has been implicated in the regulation of biofilm formation, plasmid transfer, motility and in several virulence factors (Williams et al., 2000).

The quorum sensing system of a *B. cepacia* genomovar III cystic fibrosis respiratory isolate has been identified and characterised and consists of *cepI* and *cepR* genes. This bacterial isolate, designated K56-2, synthesises *N*-octanoyl homoserine lactone (C_8-HSL) and *N*-hexanoyl homoserine lactone (C_6-HSL) (Lewenza et al., 1999; Lutter et al., 2001). The CepI/R quorum sensing system of *B. cepacia* K56-2 has been implicated in the negative regulation of the siderophore ornibactin and in positive regulation of a secreted protease. Similarly, the CepI/R quorum sensing system has been recently identified and characterised from another *B. cepacia* genomovar III cystic fibrosis respiratory isolate, designated H111, and shown to be involved in regulating biofilm formation and swarming motility (Huber et al., 2001). In addition, quorum sensing systems are present and conserved among the heterogeneous *B. cepacia* complex (Gotschlich et al., 2001).

The identification and characterization of the quorum sensing system of the environmental isolate *B. cepacia* type strain ATCC 25416 (Genomovar I), is reported in this chapter. It was observed that *cepI* was positively autoregulated by CepR in the presence of HSL. Consequently, a *cepR-PcepI-lacZ* reporter HSL-sensor plasmid was constructed and it is shown that (i) a *cepR* knock-out mutant results in a 70 % decrease in HSL production, (ii) that CepR responded to C_6-HSL with only 15% the molar efficiency of C_8-HSL, and (iii) that CepR responds best to long chain HSL autoinducers.

2.2   MATERIALS and METHODS.

2.2.1   Bacterial strains and media.

Burkholderia cepacia ATCC 25416 (or LMG 1222) is an isolate from rotten onion (Palleroni & Holmes, 1981) and was routinely grown in Luria-Bertani broth (Sambrook *et al.*, 1989), M9 minimal medium (Sambrook, *et al.*, 1989) or M9GP (Gonzalez *et al.*, 1997) at 30°C. *Chromobacterium violaceum* CVO26 is a double mini-Tn*5* mutant derived from ATCC 31532, this mutant is non-pigmented and production of the purple pigment can be induced by providing exogenous HSL inducer molecules (McClean, *et al.*, 1997). *Escherichia coli* strains used in this study included HB101 (Sambrook, *et al.*, 1989), DH5α (Hanahan, 1983), HB101::Tn5 (Magazin *et al.*, 1986) and were grown in LB medium (Miller, 1972) at 37°C. Antibiotics were added as required at final concentrations of tetracycline 10 µg/ml (*E. coli*), 20 µg/ml (*C. violaceum*), 300 µg/ml (*B. cepacia*), gentamycin 10 µg/ml (*E. coli*), 300 µg/ml (*B. cepacia*), ampicillin 100 µg/ml (*E. coli*), kanamycin 50 µg/ml (*E. coli*) and 300 µg/ml (*B. cepacia*), streptomycin 100 µg/ml (*C. violaceum*).

2.2.2   Recombinant DNA techniques.

Digestion with restriction enzymes, agarose gel electrophoresis, purification of DNA fragments, ligation with T4 DNA ligase, end filling with Klenow fragment of DNA

polymerase, Southern hybridisation and transformation of *E. coli* were performed as described by Sambrook *et al.,* (1989). Analytical amounts of plasmids were isolated as described by Birnboim (1983), whereas preparative amounts were purified with Qiagen columns (Valencia, CA, USA). Total DNA from *B. cepacia* was isolated by sarcosyl-pronase lysis as described by Better *et al.* (1983). Tri-parental matings from *E. coli* to *B. cepacia* were performed with the helper strain *E. coli* (pRK2013) (Figurski & Helinski, 1979). A genomic cosmid library of *B. cepacia* ATCC 25416 was constructed using a Gigapack III XL packaging extract kit (Stratagene, La Jolla, CA, USA), following the instructions provided by the supplier. Genomic DNA (100 µg) was partially digested with *EcoRI* and ligated into the corresponding site in pLAFR3. The formation of DNA concatamers was verified by agarose gel electrophoresis and 1 µg of this DNA was used for the packaging reaction. About 300 colonies were obtained when *E. coli* HB101 cells were infected with 5 µl of the packaging reaction. Approximately 30,000 recombinant cosmids were obtained in total.

2.2.3   Cloning of quorum sensing genes of *B. cepacia* ATCC 25416.

About 4×10^9 cells from mid-log growth phase of *E. coli* HB101 harbouring the *B. cepacia* ATCC 25416 cosmid library in pLAFR3, 4×10^9 of *E. coli* (pRK2103), and 2×10^8 cells of *C. violaceum* CVO26 were washed in LB and mixed. The suspension was applied to a 0.45 µm membrane filter (Millipore Corp.) on an LB plate. After overnight incubation at 30°C, cells were resuspended and spread on LB plates containing ampicillin (100 µg/ml), kanamycin (100 µg/ml), streptomycin (100 µg/ml) and

tetracycline (20 μg/ml). Strain CVO26 is naturally resistant to ampicillin and streptomycin, and resistant to kanamycin due to the miniTn5 present on the chromosome. Tetracycline selected transconjugants of strain CVO26 having the pLAFR3 based cosmid clone. These plates were incubated for 48 hrs at 30°C and transconjugants that turned purple were further assayed. Six cosmids (pCQS1-6) from the cosmid library could restore purple pigmentation and they shared only a 9 kb EcoRI insert. Further subcloning confirmed that the 9 kb EcoRI fragment cloned in the corresponding site in pLAFR3 (creating pLIR5) could restore pigmentation in strain CVO26. The 9 kb EcoRI was also cloned in the corresponding site in pBluescript KS+, thus creating pBIR.

2.2.4 Transposon Tn5 mutagenesis.

Transposon Tn5 insertions within recombinant plasmid pLIR5 was performed as described by Magazin *et al.* (1986) with *E. coli* HB101::Tn5 as the source of the transposon. *E. coli* HB101 cells containing Tn5 insertions within plasmid pLIR5 were identified by purifying plasmid DNA from HB101::Tn5 (pLIR5), using it to transform *E. coli* DH5α, and selecting for plasmids having tetracycline and kanamycin resistance. These recombinant plasmids in *E. coli* DH5α were delivered by triparental conjugation to *C. violaceum* CVO26 as described above. Transconjugants of strain CVO26 (pLIR5::Tn5) lacking the expression of violacein (i.e. which remained white) were further analysed (Figure 1).

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2.2.5   Construction of a *B. cepacia* ATCC 25416 *cepI* and *cepR* knock out mutants.

The kanamycin resistance gene from pSUP2021 was cloned as a 3.5 Kb *Hind*III fragment in the corresponding sites of *cepI* present in plasmid pLIR5, thus obtaining pLCIKm (Figure 2a). Plasmid pLIR::Tn*5*3 (contains a Tn*5* insertion in the *cepR* gene harboured in pLAFR3) and plasmid pLCIKm (contains a Km resistance gene cloned in the *cepI* gene) were recombined with the corresponding target regions of the genome of *B. cepacia* ATCC 25416 by a marker exchange procedure (Corbin *et al.,*1982; Kojic & Venturi, 2001). Plasmid pPH1JI was used as the incoming IncP1 incompatible plasmid and selections were made on LB plates containing kanamycin and gentamycin. This generated two genomic mutants designated *B. cepacia* 25416-I and *B. cepacia* 25416-R, harbouring a Km cassette in the *cepI* gene and a Tn*5* insertion in the *cepR* gene respectively. The fidelity of each marker exchange event was confirmed by Southern analysis (Figure 2b).

2.2.6   Construction of a *B. cepacia* CepR based HSL detecting plasmid.

The *cep* genes of *B. cepacia* ATCC 25416 were used to construct a plasmid in order to detect HSL molecules in a heterologous bacterial background. A *Bam*HI-*Hind*III DNA fragment of 3.5 kb from pBIR containing the whole *cepR* gene and the *cepI* promoter together with the first 120 bp of *cepI* were inserted into the corresponding sites of the vector pQF50 (Farinha & Kropinski, 1990) yielding pSCR1. Plasmid pQF50 contains a

promoterless *lacZ* gene and in plasmid pSCR1 the transcription of the *lacZ* gene is under the control of the *cepI* promoter (Figure 4).

2.2.7   Reporter gene fusion assay.

β-galactosidase activity was determined as described by Miller (1972) with the modifications of Stachel *et al.* (1985).

2.2.8   Purification, detection and visualisation of autoinducer (HSLs) molecules.

The purification, detection and visualisation of HSLs molecules from culture supernatants were performed as described by McClean *et al.* (1997) and Kojic & Venturi (2001). *C. violaceum* CVO26 was used as the indicator strain on TLC plates detecting the presence of HSL molecules (McClean, *et al.*, 1997). Synthetic HSLs (C_4-HSL, C_6-HSL, C_7-HSL, C_8-HSL, C_{10}-HSL, C_{12}-HSL) were purchased from Fluka Chemie AG (Buchs, CH). For quantification of CepR activity, overnight *E. coli* DH5α (pSCR1) cultures were normalised to an O.D. 600 nm of 0.1 in a volume of 20 ml LB containing the desired HSL at the desired concentration. Cultures were then grown with agitation at 37°C for 6 hrs and β-galactosidase activities were determined. Presence of HSL was also detected on solid media by growing *E. coli* (pSCR1) on X-gal and ampicillin media in close proximity to the tester strain. Presence of HSL was observed when *E. coli* (pSCR1) turned blue (Figure 4).

2.2.9   DNA sequence determination, analysis and database accession number.

The DNA sequence of the *cepI/R* locus was determined using pBIR as template. Nucleotide sequences were determined by the dideoxy chain-termination method (Sanger *et al.*, 1977) using [^{35}S]dATP for labelling and 7-deaza-dGTP (Pharmacia) instead of dGTP. Analysis of the sequence was performed with EditSeq and MapDraw software (DNASTAR, Inc.). BLASTX and BLASTN programs were used to search the non-redundant sequence database for homologous sequences (Madden *et al.*, 1996). The sequence of *cepI-cepR* locus has been deposited in the GenBank/EMBL/DDBJ database under accession numbers AJ422183.

2.2.10 Plasmids used.

Table1. Plasmids used in this study.

Plasmid	Description	Source or refrence
pBluescript KS	Ap^r; ColE1 replicon	Stratagene
pUC18	Ap^r; ColE1 replicon	(Yanisch-Perron *et al.*, 1985)
pMP190	Cm^r; IncQ; promoter probe vector	(Spaink *et al.*, 1987)
pUC4K	Ap^r; pBR322 replicon	Amersham Pharmacia
pMP77	Cm^r; IncQ; promoter probe vector	(Spaink, *et al.*, 1987)
pRK2013	Km^r;Tra$^+$; Mob$^+$; ColE1 replicon	(Figurski & Helinski, 1979)
pPH1Jl	Gm^r; IncP1	(Beringer *et al.*, 1978)
pSUP2021	Ap^r; Cm^r; Tc^r; Km^r; pBR325 replicon; Broad-host-range vector	(Simon *et al.*, 1983)
pLAFR3	Tc^r; Broad-host-range cloning vector; IncP1	(Staskawicz *et al.*, 1987)
pQF50	Ap^r; Broad-host-range vector; pRO1600 replicon	(Farinha & Kropinski, 1990)
pLIR5	Tc^r; pLAFR3 containing *cep*IR locus in a 9 Kbp *EcoR*I fragment	This study
pBIR	Ap^r; pBluescript KS containing *cepIR* locus in a 9 Kbp *EcoR*I fragment	This study
pCQS 1-6	Tc^r; pLAFR3 containing *B. cepacia* DNA	This study
pLIR::Tn53	Tc^r; Km^r; pLIR5 with a Tn5 insertion in *cepR*	This study
pLCIKm	Tc^r; Km^r; pLIR5 with a Km cassette in *cepI*	This study
pSCR1	Ap^r; pQF50 containing P*cepI-lacZ* and *cepR*	This study
pMPIR	Cm^r; pMP77 containing the *cepIR* locus in a 4.5 Kbp *PstI* fragment	This study

^{a} Ap^r, Cm^r, Gm^r, Km^r, Tc^r: resistant to ampicillin, chloramphenicol, gentamycin, kanamycin and tetracycline, respectively.

2.3   RESULTS.

2.3.1   Identification of the quorum sensing system of *B. cepacia* ATCC 25416.

The quorum sensing systems of two clinically isolated strains of *B. cepacia* (belonging to genomovar III) have been identified and associated for their role in siderophore production and ability to form biofilms (Huber, *et al.*, 2001; Lewenza, *et al.*, 1999). In the present study, the quorum sensing system of the environmental isolate *B. cepacia* ATCC 25416 belonging to genomovar I, was investigated. It has been previously reported that this strain produces both C_8 and C_6-HSL (Gotschlich *et al.*, 2001). In agreement with this previous report, organic extraction from overnight spent supernatants were developed by TLC and visualised with the CVO26 biosensor, confirming that these molecules are synthesised by ATCC 25416 (Figure 1, lane 2). In addition, it was also possible to observe that there is another faint signal in between the C_8- and C_6-HSL, possibly corresponding to C_7-HSL (Figure 1, lane 2; Gotschlich *et al.*, 2001).

The genomic locus coding for the quorum sensing genes of ATCC 25416 was identified using the biosensor strain *Chromobacterium violaceum* CVO26. *C. violaceum* is a naturally purple pigmented Gram-negative bacteria, and the pigmentation phenotype is regulated by quorum sensing. CVO26 is a double mini-Tn5 mutant derived from the parent strain ATCC 31532, where the *cviI*, coding for the autoinducer synthase of the cognate HSL (C_6-HSL) is interrupted. Pigmentation can be restored by exogenously

providing HSLs (McClean, *et al.*, 1997). A cosmid library of *B. cepacia* ATCC 25416 was constructed in pLAFR3 and introduced by triparental conjugation in *C. violaceum* CVO26 (see Materials and Methods for details). After selection on appropriate growth plates, six colonies of *C. violaceum* CVO26 were found to be purple pigmented. These colonies were isolated for further analysis. Cosmid DNA was extracted and used to transform *E. coli* DH5α for restriction enzyme analysis. DNA digestions with *EcoR*I revealed that all six cosmids (pCQS1-6) shared only a 9 kb common insert. Further subcloning confirmed that the 9 kb *EcoR*I fragment cloned in pLAFR3 (creating pLIR5) could restore pigmentation in strain CVO26. TLC analysis using extracts from spent supernatant of strain CVO26 (pLIR5) show that C_6- and C_8-HSL were now produced (Figure1, lane 5). It was postulated that this DNA fragment contained the necessary information to synthesise the autoinducer molecules of *B. cepacia* ATCC 25416.
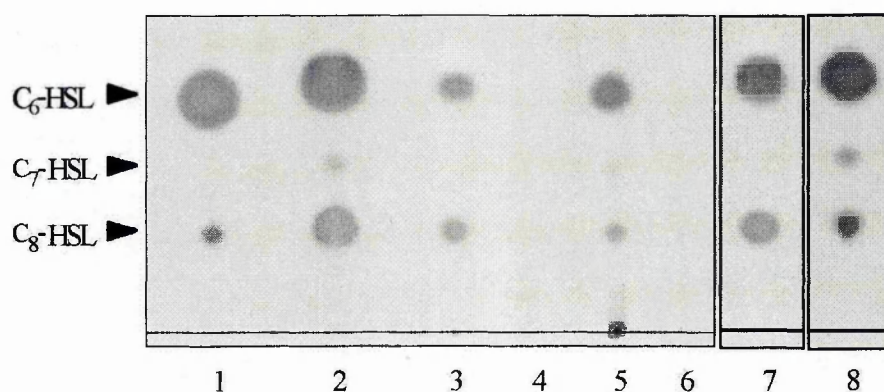
Figure 1. Analytical TLC of *B. cepacia* and *C. violaceum* CVO26 spent supernatant extracts. Acyl-homoserine lactones (HSLs) were extracted from 10 ml of spent supernatants, spotted onto a C_{18} reverse-phase TLC plate and developed with a methanol-water (60/40, vol/vol) solvent mixture. HSLs were visualised using an overlay of agar seeded with *C. violaceum* CVO26. Lane 1 are C_6-HSL and C_8-HSL synthetic standards; Lane 2 from spent supernatant of *B. cepacia* ATCC 25416; Lane 3, from spent supernatant of *B. cepacia* 25416-R; Lane 4, from spent supernatant of *B. cepacia* 25416-I; Lane 5, from spent supernatant of *C. violaceum* CVO26 (pLIR5), Lane 6, from spent supernatant of *C. violaceum* CVO26 (pLIR5::Tn53), Lane 7 from spent supernatant of *B. cepacia* 25416-I (pMPIR) and Lane 8 from spent supernatant of *B. cepacia* 25416-R (pMPIR).

2.3.2   Characterisation of the quorum sensing system of *B. cepacia* ATCC 25416

In order to further study the 9 kb *Eco*RI fragment, containing the necessary information to allow pigmentation in strain CVO26 (see above), this fragment was cloned in the ColE1 based plasmid replicon pBluescript KS, yielding pBIR. Restriction enzyme analysis showed the presence of three *Pst*I fragments of 4500, 2500 and 2000 bp each. DNA sequence analysis of the two latter fragments revealed the presence of two ORFs, designated *cepI* and *cepR*, coding for a LuxI and a LuxR homologue respectively. The *cepI* ORF is divergently transcribed from *cepR* with an intergenic region of 727 bp. A genetic map of the locus coding for these two genes is depicted in Figure 2a. The genomic *cepI* gene coded for a protein of 202 amino acids with a predicted molecular weight of 22.2 kDa and the *cepR* coded for a protein of 239 amino acids with a predicted molecular weight of 26.6 kDa. The highest amino acid identity of CepI and CepR was with the homologues of previously identified *B. cepacia* genomovar III isolates K56-2 and H111 (Huber, *et al.*, 2001; Lewenza, *et al.*, 1999) displaying approximately 98 % identity. Table 2 shows the CepI and CepR amino acid identity of the different members of the *B. cepacia* complex, in relation to ATCC 25416.

Table 2. Amino acid CepIR identity between different members of the *B. cepacia* complex.

Genomovar	Species or strain	CepI[a]	CepR
Genomovar I	*B. cepacia* ATCC 17759	97	94
Genomovar II	*B. multivorans* ATCC 17616	78	94
Genomovar III	*B. cepacia* K56-2	98	97
	B. cepacia H111	98	97
Genomovar IV	*B. stabilis* LMG 14291	97	95
Genomovar V	*B. vietnamiensis* R-921	91	96
	B. vietnamiensis PC259	97	97
Genomovar VI	*B. cepacia* LMG18943	92	93
Genomovar VII	*B. ambifaria*	96 (BafI)	93 (BafR)

[a]Value expressed as a percentage of amino acid identity to CepI and CepR from *B. cepacia* ATCC 25416.

The *cepI* promoter region of ATCC 25416 was compared to the corresponding regions present in strain K56-2, *B ambifaria* and *B. multivorans*. As can be seen in Figure 3a, the putative *cepI* promoter elements −35 and −10 are identical in all four strains. The putative *lux*-type boxes are also identical, with only the exception of the first two nucleotides of *B. ambifaria* and *B. multivorans*.

Interestingly, the region upstream of *cepR* contained a 399 bp open reading frame (ORFX, Figure 2a), which was preceded by a putative σ^{70}-like promoter.
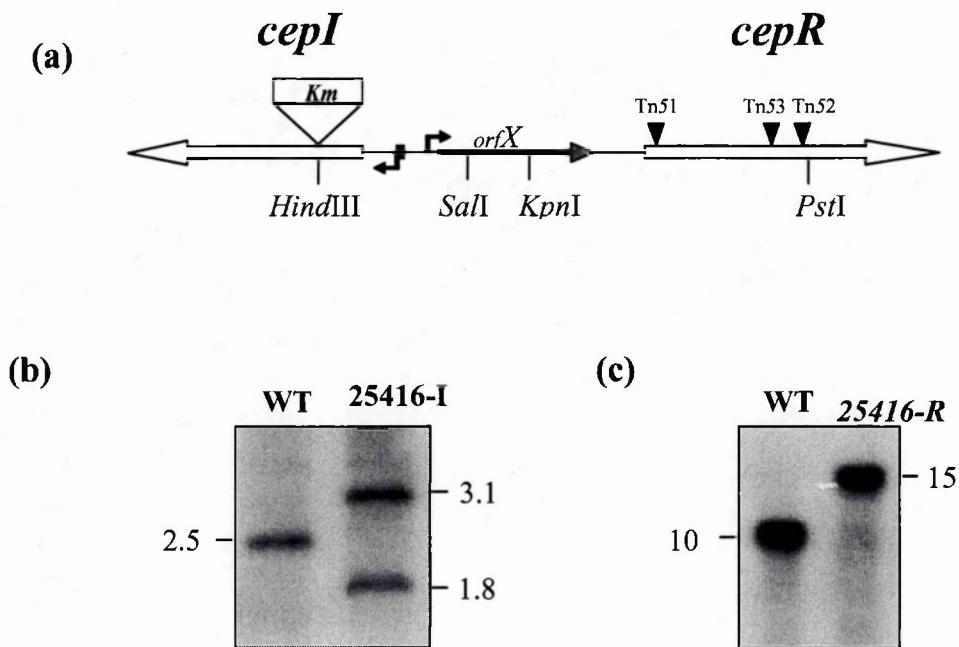
Figure 2. The *cepI/R* locus (a)Gene map of the *cepI/R* locus of *B. cepacia* ATCC 25416. Shown are the three Tn*5* insertions in *cepR* and the position of the Km cassette cloned in *cepI* (see Materials and Methods for details). Black arrows indicate the promoter of *cepI*, in front of the putative *lux*-type box (small rectangle); the putative promoter of *cepR* is located in front of the ORFX. **(b) Construction of 25416-I as verified by Southern analysis.** The *cepI* gene in the chromosome was replaced with the cloned *cepI*::Km as described in the text. Lane 1 is genomic DNA digested with *Pst*I and lane 2 is genomic DNA from 25416-I digested with *Pst*I. These were then probed with a PCR generated fragment containing the complete sequence of *cepI*. In the parent strain (lane 1) a fragment of 2.5 kb was observed whereas in the mutant this fragment is split in two due to the *Pst*I site present in the Km cassette. **(c) Construction of 25416-R as verified by Southern analysis.** The *cepR* gene in the chromosome was replaced with the cloned *cepR*::Tn53 as described in the text. Lane 1 is genomic DNA digested with *Eco*RI and lane 2 is genomic DNA from 25416-R digested with *Eco*RI. These were then probed with a 2,5 Kb *Pst*I fragment containing *cepR*. In the parent strain (lane 1) a fragment of approximately 10 kb was observed whereas in the mutant this fragment was approx 15 kb, due to the presence of Tn*5* in the *cepR* gene.

A BLAST search of current public databases using the derived amino acid sequence of ORFX of the *cepI/R* locus as query sequence, failed to reveal any closely related protein that might suggest a function for the ORFX product. Sequence comparison of this promoter region between some members of the *B. cepacia* complex is shown in Figure 3b.

(a)

lux-type box

B. ambifaria	ACCTGTAAGAGTTACCAGTTACCGCCTCCTCGGGACGCGCGCTGTAATGCGTGCATACAA
B. multivorans	TACTGTAAGAGTTACCAGTTACCGCCGCCTCGGGACGCGCGCTGTAATGCACGCATACCA
ATCC25416	CCCTGTAAGAGTTACCAGTTACAGCCCCCTCGTGCGGCGCGATGTAATGCGCACATACAA
K56-2	CCCTGTAAGAGTTACCAGTTACAGGCTCCTCGTGCCGCGCGCTGTAATGCACGCATACAA
	****************** * * ***** * ***** ******** ***** *
	-35 -10

B. ambifaria	AG--ATCAATCCGAGGACACCCATGCGAACCTTCGTT
	M R T F V
B. multivorans	AA--ACCCATCCGAGGACATCCATGCAGACCTTCGTT
	M Q T F V
ATCC25416	AAGCACAAATCCGAGGACA-CCATGCGGACCTTCGTT
	M R T F V
K56-2	AAGCACAGATCCGAGGACATCCATGCAGACCTTCGTT
	* * ********** **
	SD **M** R T F V

(b)

ATCC25416	TTGAAAACCGGGCCGCTTTTGCTTATAAAGAGCGCCAGCACTGCGGAAATTGTCAGAGGAATGCGGGGCCAGTTG
	M R G Q L
K56-2	TTGAAAACCGCGCCGCTTTTGCTTATAAAGAGCGCCAGTCTTGCA-AAATCGTCAGAGGAATGCGCGGCCGGTTG
	M R G R L
B.multivorans	TTGAAAACGCGCGCGCTTTTGCTTATAAAGAGCGCCGGAATCGCGGAAAC-GTCAGAAGAATGCAGCGGTTGTCA
	M Q R L S
B.ambifaria	TTGAAATACGGGCGGCTTTTGCTTATAAAGAGCGCCAGTCTTGAGGAAAT-GTCAGAGGAAAGCGTGGCAGATTGT
	****** ******************** * * *** ******** **V** A D C
	-35 -10 SD

Figure 3. Nucleotide sequence of *luxI* and ORFX promoter region **(a)** Sequence comparison of the putative promoter elements present upstream of the LuxI homologues in four members of the *B. cepacia* complex. The first amino acids of the respective *luxI* homologues are also shown. **(b)** Sequence comparison of the ORFX upstream region. The first amino acids of the putative protein encoded by this ORF are also shown.

2.3.3 Construction of insertionally inactivated *cepI* and *cepR* genes in *B. cepacia* ATCC 25416.

In order to investigate the role of quorum sensing in *B. cepacia* ATCC 25416, the *cepI* and *cepR* genes were inactivated in the chromosome. Tn*5* mutagenesis was performed on pLIR5, producing three independent insertions, thus generating pLIR5::Tn*51*-3, which abolished the ability of pLIR5 to either restore pigmentation or to synthesise any detectable levels of HSL in strain CVO26 (see Materials and Methods and Figure 1, lane 6). Further DNA restriction and sequence analysis revealed that all three insertions were located in different positions in the *cepR* gene (Figure 2a). Plasmid pLIR5::Tn*53* was used in a marker exchange experiment in order to introduce insertion mutations site-specifically within the *cepR* gene of strain ATCC 25416. This resulted in the construction of a *cepR*::Tn*5* knock-out mutant called *B. cepacia* 25416-R. Similarly, plasmid pLCIKm, a pLIR5 containing a Km resistance cassette cloned in the *cepI* gene, was used in a similar experiment resulting in a *cepI*::Km knock-out mutant called *B. cepacia* 25416-I (Figure 1, lane 4 and Figure 2a). The fidelity of the marker exchange event for both mutants was confirmed by Southern analysis (Figure 2b,c).

2.3.4 CepR responds best to C$_8$-HSL and regulates HSL production.

On plate assays and on TLC using biosensor strain CVO26, it was observed that the *cepR* mutant of strain ATCC 25416 produced far less HSL molecules when compared to the wild type (Figure 1, lane 3). In order to more precisely quantify the HSL production in *B. cepacia*, a *cep*-based HSL detecting plasmid was constructed. This sensor plasmid,

designated pSCR1, contains the complete *cepR* gene and intergenic region, with the promoter and the first 120 nucleotides of *cepI* transcriptionally fused with a promoterless *lacZ* gene (Figure 4a).

It was observed that on solid media containing X-gal, *E. coli* DH5α (pSCR1) could conveniently be used to detect the production of HSL when streaked in close proximity to tester strains (Figure 4b). Since *B. cepacia* ATCC 25416, like many other strains of the *B. cepacia* complex, synthesises C_6- and C_8-HSL (Gotschlich, *et al.*, 2001), the ability of CepR to activate transcription using these two HSLs was tested using pSCR1. This was performed by measuring β-galactosidase activity of *E. coli* DH5α (*E. coli* DH5α is known not to produce HSL-type signal molecules) harbouring pSCR1, grown in 20 ml LB containing either C_6- or C_8-HSL at increasing concentrations.

As depicted in Figure 5, CepR responded to C_6-HSL with only 15% the molar efficiency of C_8-HSL; the C_8-HSL molecule was detected at concentrations lower than 10 nM and the reporter plasmid reached its highest β-galactosidase activity when 100 nM of the molecule was present. Higher concentrations did not result in higher β-galactosidase enzyme activity. The activity of CepR in presence of C_6-HSL was low even at concentrations as high as 1000 nM of C_6-HSL. No significant β-galactosidase activity was detected when HSLs were not added to the culture.

(a)

Pcep1

cepR   lacZ   bla   pSCR1

T₀

(b)

E.coli (pSCR1)   *E.coli* (pQF50)

B. cepacia ATCC 25416
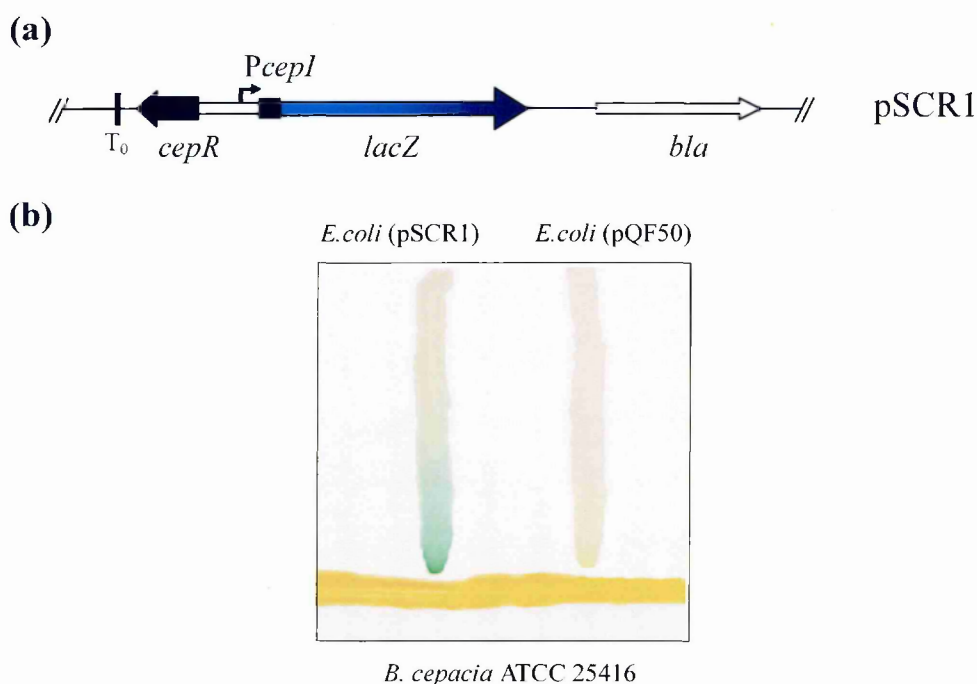
Figure 4. The pSCR1 biosensor (a) Schematic diagram (not to scale) of the *cep*-based HSL sensor plasmid used in this study. Plasmid pSCR1 contains a *cepI–lacZ* transcriptional fusion together with the *cepR* gene cloned in the broad-host-range plasmid pQF50. The genetic components are: P*cepI*, promoter of the HSL synthase of ATCC 25416; *cepR*, gene encoding the transcriptional activator CepR; *lacZ*, β-galactosidase gene; T₀, transcriptional *trpA* terminator; bla, ampicillin-resistance marker. **(b)** Activation of bacterial sensor strain in cross-streak experiments. The sensor strain, *E. coli* DH5α (pSCR1) was cross-streaked in LB agar plates (containing ampicillin and X-gal) using *B. cepacia* ATCC 25416 as tester strain. In closer proximity to the tester strain, the sensor became blue. No colour was observed when the plasmid pQF50 was used instead of pSCR1.

As described above, analytical TLC shows that there is less production of HSL in 25416-R than the wild type (Figure 1a, lane 3). In order to precisely determine this difference, spent supernatants from both 25416-R and ATCC 25416 were extracted and further assayed with *E. coli* (pSCR1). When spent supernatants of 25416-R were assayed with *E. coli* (pSCR1), it was possible to determine that 24516-R produces approximately 30 % of the HSLs produced by the wild type parent strain. The HSL production was restored when the mutant of *B. cepacia* 25416-R was complemented with pMPIR (Figure 1, lane 8). Taken together, these results suggest that the system needs CepR in order to maximally express *cepI* for HSL production. Furthermore, based on the β-galactosidase activity obtained, it was estimated that a culture of *B. cepacia* ATCC 25416 at O.D. 600 nm of 1 contains approximately 100 nM of C_8-HSL.

2.3.5   CepR responds better to long chain HSLs.

The different HSL bacterial biosensors described so far in the literature elicit a degree

of response that depends on the nature of the HSL to be tested. For this reason, the

response of pSCR1 to different HSL molecules was analysed. This was done by

measuring β-galactosidase activity of *E. coli* DH5α (pSCR1) exposed to 100 nM HSL

molecules of different acyl chain length. It was decided to use 100 nM since this was

the minimum concentration of the C_8-HSL in order to achieve a good response wuth the

sensor (Figure 5a). As can be seen on Figure 5b, the CepR activity was about 80 % with

the long chain (C_{10} and C_{12}), whereas it was only about 30 % with the short chain (C_4,

C_6, C_7) HSLs, when compared to the activity obtained with C_8-HSL. Taken together,

these results suggest that, in an *E. coli* background, CepR is more active with long chain

HSLs.
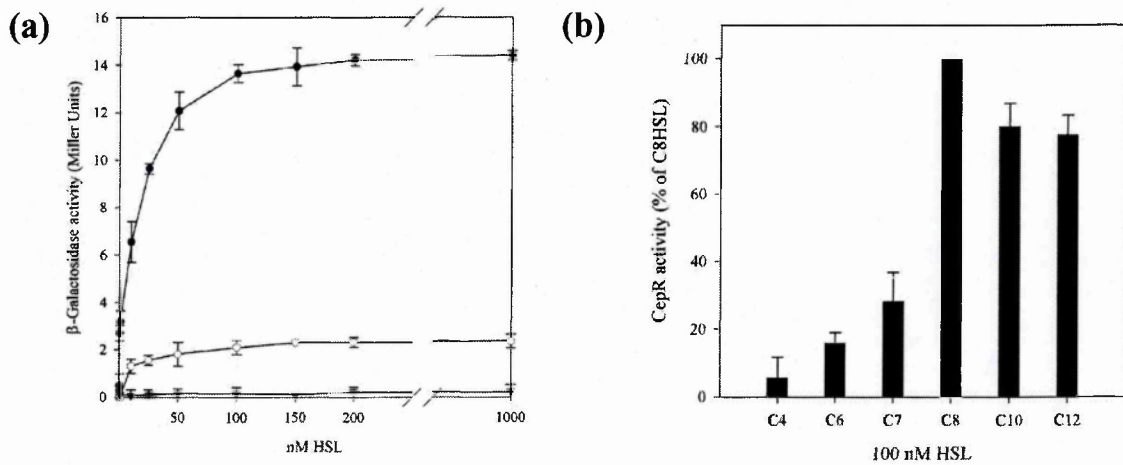
Figure 5. CepR activity for different HLSs **(a)** HSL bioassay using *Escherichia coli* DH5α (pSCR1). DH5α (pSCR1) was grown in the presence of varying concentration of either C_6-HSL (○), C_8-HSL (●) or no HSL (▼); β-galactosidase activities were determined after six hours. The values were determined with LB medium and the means of triplicate experiments are given, the standard deviation is shown. **(b)** CepR-HSL response. *E. coli* DH5α (pSCR1) was grown in the presence of 100 nM of either C_4-, C_6- C_8- C_{10}- and C_{12}-HSL and β-galactosidase activities were determined after six hours. The values were determined with LB medium and the means of triplicate experiments are given, the standard deviation is shown. The values are expressed as a percentage of the activity determined using C_8-HSL.

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2.4   DISCUSSION.

Strains of *Burkholderia cepacia* have recently been sub-divided into a group of nine distinct genomovars (Vandamme, *et al.*, 2002). In the present study, the genetic determinants coding for the quorum sensing system in the environmental isolate *B. cepacia* type strain ATCC 25416, belonging to Genomovar I, were identified and characterised. The quorum sensing system was composed of the *cepI* and *cepR* genes, which were found to be genetically linked and highly similar to the recently isolated CepI/R of two Gv. III *B. cepacia* clinical isolates (Huber, *et al.*, 2001; Lewenza, *et al.*, 1999; Lutter *et al.*, 2001).

While the present studies were in progress, several representative strains belonging to the *B. cepacia* complex were analysed by others for the presence of the *cep* loci (Gotschlich, *et al.*, 2001). Southern hybridisation experiments show that the *cep* locus is present in all the tested strains; in addition, PCR amplification of the respective *cepI* and *cepR* homologues show that there is a very high degree of conservation between the different strains. The *cepI* and *cepR* genes were in all cases genetically linked, divergently transcribed, and with an intergenic region between them (Gotschlich, *et al.*, 2001).

2.4.1   Characterisation of the *B. cepacia* ATCC25416 quorum sensing system

In the present study, a genomic cosmid clone coding for the *cepI/R* locus was isolated by its ability to restore pigmentation in the sensor strain *C. violaceum* CVO26. It was

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observed that the interruption of the cloned *cepR* by transposon insertion mutagenesis dramatically reduces the HSL produced via CepI in strain CVO26 (Figure 1, lane 3 and see below). This phenomenon could be explained by positive autoregulation of *cepI* by CepR and HSL. Interestingly, promoter analysis of the *cepI* gene revealed a putative *lux*-type box partially overlapping the putative −35 site of the promoter (Figure 3a), thus possibly allowing signal amplification via positive autoregulation. This observation is in accordance to what occurs in *B. cepacia* K56-R2 (Lewenza, *et al.*, 1999) and in other related systems such as in *Pseudomonas aeruginosa*, where LasR (bound to 3-oxo-C_{12}-HSL) activates *lasI* (de Kievit *et al.*, 2002; Seed *et al.*, 1995), in *V. fischeri*, where LuxR (bound to 3-oxo-C_6-HSL) activates *luxI* (Engebrecht & Silverman, 1984; Engebrecht & Silverman, 1987), and in *Ralstonia solanacearum*, where SolR (bound to C_8-HSL) activates *solI* (Flavier, *et al.*, 1997b).

It has been previously shown that *B. cepacia* produces C_6-HSL and C_8-HSL (Gotschlich, *et al.*, 2001). Here it is shown that an additional molecule is able to induce pigmentation in strain CVO26 when cell-free organic extracts of *B. cepacia* were spotted and developed on TLC. This signal migrates between the C_6-HSL and C_8-HSL and corresponds presumably to C_7-HSL (Figure 1). A similar signal was detected in extracts of *E. coli* expressing *cepI* from *B. cepacia*, and was identified possibly as C_7-HSL by comparison with standard molecules on TLC (Gotschlich, *et al.*, 2001). The presence of a C_7-HSL is intriguing since it is unusual to find HSL with an odd number of acyl chain carbon atoms, and has only been previously chemically confirmed in *Rhizobium leguminosarum* (Lithgow *et al.*, 2000) and *Serratia marcescens* (Horng *et al.*, 2002). The use of propionyl-CoA and malonyl-CoA as acyl chain starter and

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extender units could result in the formation of precursors for the acyl chain in which an

odd number of carbon atoms are present (Horng *et al.*, 2002).

2.4.2   Sequence analysis of the promoter and the intergenic region.

It was possible to localize a putative *lux*-type box, a putative −35 and −10, and a Shine

Dalgarno consensus region in the promoter of *cepI* (Figure 3). The putative *lux*-type box

sequence was compared with *cepI* promoters present in other members of the *B. cepacia*

complex and it was observed that there is a high percentage of nucleotide identity

(Figure 3a). However, *B. ambifaria* (Genomovar VII) and *B. multivorans* (Genomovar

II) have one and two bases different to ATCC 25416 and K56-2 in the putative *lux*-type

box respectively. It would be interesting to further study the functionality of these

putative *lux* boxes and also verify if these small differences in nucleotide sequence

mean a different degree of *cepI* regulation. In addition, cross-complementation studies

of the *cepR*-null mutants of different *B. cepacia* strains could reveal if the small

differences in primary structure of the CepR proteins and of the *cepI* promoters

observed are significant.

The intergenic region present between *cepI* and *cepR* has another interesting feature. A

putative 399 bp ORF was found, similar to what was observed upstream of *cepR* in *B.*

cepacia K56-2 (Genomovar III), *B. multivorans* ATCC 17616 and of *bafR* in *B.*

ambifaria (Figure 3b). A BLAST homology search did not reveal any closely related

protein that might suggest a function for this ORF in ATCC 25416 or *B. multivorans*

and *B. ambifaria* (Yao *et al.*, 2002). It is noteworthy that this ORF is preceded by a

putative σ^{70}-like promoter, which may possibly drive also the expression of the downstream *cepR* gene. No studies have been directed to verify any function of this ORF and whether it could be another regulatory element in the *cepIR* locus.

2.4.3   Characterisation and usefulness of a *cep* based biosensor.

As mentioned above, *cepI* is positively regulated by CepR bound to its cognate HSL. Taking advantage of this feature, a sensor plasmid (pSCR1), which is made of components of the *cep* quorum sensing system of strain ATCC 25416, was constructed. This sensor plasmid contains the promoter of *cepI* transcriptionally fused to a promoterless *lacZ* reporter gene together with the *cepR* gene in a wide host range plasmid. The resulting plasmid based biosensor was used in an *E. coli* genetic background and it was determined that *B. cepacia* 25416-R produces approximately 70 % less HSLs than the wild type strain, thus further confirming the observation of a reduced HSL production on TLC (Figure 1, lane 3). It was also concluded that on solid media containing X-gal, pSCR1 harboured in *E. coli* could conveniently be used to detect the production of HSL when streaked in close proximity to a tester strain (Figure 1a).

2.4.4   CepR responds best to C$_8$-HSL.

The quorum sensing system of *B. cepacia* ATCC 25416 was characterised in more detail using the pSCR1 biosensor in *E. coli*. Increasing concentrations of C$_6$-HSL and

C_8-HSL were tested with *E. coli* (pSCR1). It was determined that 100 nM C_8-HSL was the concentration needed to maximally activate the biosensor and consequently, CepR (Figure 5a). Surprisingly however, C_6-HSL results in a very low response, thus most probably meaning a poor interaction of this molecule with CepR (Figure 5a). This is not fully explainable with the experiments shown here and it questions the function of this molecule in *B. cepacia*.

In other bacteria, the presence of HSL molecules at low concentration has also been described. For example in *P. aeruginosa*, it was shown that the LuxI homolog RhlI, is able to recognise two acyl-ACP, hexanoyl-ACP and butyryl-ACP resulting in the biosynthesis of two HSLs, C_4-HSL and C_6-HSL. However, the ratio of the HSLs products, C_4-HSL and C_6-HSL, were 15:1 in vivo and 20:1 in vitro, thus suggesting that the cellular levels of hexanoyl-ACP are not limited relative to butyryl-ACP levels and that RhlI is able to discriminate between the acyl-ACPs (Parsek, *et al.*, 1999). It is possible therefore that the CepI kinetics is similar to RhlI; further experiments with a purified CepI could possibly answer to this question.

In *B. cepacia*, C_8-HSL is the major HSL found in the supernatant (Lewenza, *et al.*, 1999) and the ratio between C_8-HSL and C_6-HSL is about 10:1, but it can vary in the different strains of the *B. cepacia* complex (Gotschlich, *et al.*, 2001). Noteworthy, no signal was detected in the *cepI*-deficient derivative of *B. cepacia* 25416-I (Figure 1, lane 4), and this is strong evidence that CepI is required for the synthesis of all the HSLs detected (C_6, C_7, and C_8-HSLs). However, it cannot be excluded that in the genome of *B. cepacia* there is another *luxR* family gene coding for another LuxR family protein

able to respond to C_6-HSL and, in this case, this molecule may possibly have a biological function.

2.4.5   CepR responds better to long chain HSLs.

The response of different acyl-chain length HSLs was tested and it was determined that the long chain HSLs elicit a better response in *E. coli* (pSCR1) than the short chain ones (Figure 5b).

An important feature when testing the response of a biosensor to different HSL or HSL analogs is the overexpression of the LuxR family protein, which can alter the results in a significant way (Zhu *et al.*, 1998). An example is with 3-hydroxy-C_4-HSL, which cannot activate *lux* genes in *V. fischeri*, but it does in an *E. coli* background (Sitnikov *et al.*, 1995). Similarly, C_{10}-HSL can be an antagonist of *lux* genes in *V. fischeri*, but an agonist in *E. coli* (Sitnikov, *et al.*, 1995). These discrepancies can be attributed to an overexpresion of LuxR. Since the β-galactosidase activities obtained in the present study with the pSCR1 biosensor were very low, CepR is unlikely to be over expressed in the *E. coli* background. Another aspect that should be considered is the bacterial host of the sensor plasmid. It has been recently shown that a sensor plasmid can give a good response with nanomolar concentrations of a certain HSL in a *Serratia liquefaciens* background, while the same sensor plasmid was only weakly stimulated at micromolar concentrations of the same HSL in a *Pseudomonas putida* background (Steidle *et al.*, 2001). Consequently, it is possible that the response of the sensor plasmid to the different HSL tested in the *E. coli* background does not represent exactly what is

happening in *B. cepacia*. Nevertheless, it was possible to determine that the sensor, *E. coli* (pSCR1), was maximally activated with C_8-HSL, and this is in good agreement with the fact that this HSL is the major autoinducer signal produced by *B. cepacia* (Lewenza, *et al.*, 1999), found in a ratio of 10:1 with respect to C_6-HSL (Gotschlich, *et al.*, 2001).

On other hand, the presence of C_7-HSL might affect slightly the estimation in *E. coli* (pSCR1) that 100 nM of C_8-HSL is regarded as the minimum concentration to maximally activate the biosensor (Figure 5a). In a *B. cepacia* background, this could be different due to the presence of C_6 and C_7 molecules, which could interfere with CepR activity either in an agonistic or antagonistic way.

IDENTIFICATION AND CHARACTERISATION OF QUORUM
SENSING-REGULATED GENES IN *Burkholderia cepacia* ATCC
25416

3.1   INTRODUCTION

As described and discussed in the previous chapters, quorum sensing is a genetic tool

that bacteria use in order to correlate the expression of certain genes to high cell density.

It is now evident that diverse Gram-negative bacteria use quorum sensing to modulate a

variety of physiological processes. Some examples include bioluminescence in *V.*

fischeri (Engebrecht & Silverman, 1987), swarming in *S. liquefaciens* (Eberl, *et al.*,

1996), antibiotic biosynthesis in *Pseudomonas* (Laue, *et al.*, 2000; Wood, *et al.*, 1997),

biofilm development in *Aeromonas hydrophila* (Lynch, *et al.*, 2002) and conjugal

transfer in *Agrobacterium tumefaciens* (Fuqua & Winans, 1994). On the other hand,

many genes have been reported to come under the control of quorum sensing, defining

in some bacterial species a quorum sensing regulon. Examples of such genes are *lasA*,

lasB, *aprA* and *toxA* in *P. aeruginosa* (Passador, *et al.*, 1993; Storey *et al.*, 1998) or

qsrP, *ribB*, *acfA*, *qsrV* and *qsr7* in *Vibrio fischeri* (Callahan & Dunlap, 2000).

In this chapter, the quorum sensing regulon of *B. cepacia* ATCC 25416 was further

characterised. Several phenotypes were tested in order to verify the role of quorum

sensing in their regulation. It was found that quorum sensing does not modulate the

expression of chitinase, siderophores or swarming ability. *B. cepacia* strain ATCC

25416 was originally isolated as an onion pathogen causing onion sour skin

(Burkholder, 1950). Onion maceration experiments have determined that quorum

sensing is involved in this pathogenicity of *B. cepacia*. The extracellular enzyme

polygalacturonase (PehA) of this strain has been previously described as an important

determinant for the maceration of onions (Gonzalez, *et al.*, 1997) and in this chapter, it

is shown that *B. cepacia* ATCC 25416 uses quorum sensing to positively modulate the expression of PehA in order to exert its pathogenicity. In addition, this chapter describes a series of experiments aimed to identify genes controlled by quorum sensing in *B. cepacia* ATCC 25416. The description of several putative quorum sensing regulated promoters and their corresponding putative gene(s) is discussed.

3.2   MATERIALS and METHODS

3.2.1   Bacterial strains and media.

B. cepacia ATCC 25416 (or LMG 1222) is an isolate from rotten onion and was grown in

Luria-Bertani broth (Sambrook et al., 1989), M9 minimal medium (Sambrook, et al.,

1989) or M9GP (Gonzalez, *et al.*, 1997) at 30°C. *E. coli* strains used in this study

included HB101 (Sambrook, *et al.*, 1989), DH5α (Hanahan, 1983), and were grown in

LB medium (Miller, 1972) at 37°C. Antibiotics were added as required at final

concentrations of tetracycline 10 μg/ml (*E. coli*), 300 μg/ml (*B. cepacia*), gentamycin 10

μg/ml (*E. coli*), 300 μg/ml (*B. cepacia*), ampicillin 100 μg/ml (*E. coli*), kanamycin 50

μg/ml (*E. coli*), 300 μg/ml (*B. cepacia*), chloramphenicol 25 μg/ml (*E. coli*), and 250

μg/ml (*B. cepacia*).

3.2.2   Recombinant DNA techniques.

Digestion with restriction enzymes, agarose gel electrophoresis, purification of DNA

fragments, ligation with T4 DNA ligase, end filling with Klenow fragment of DNA

polymerase, Southern hybridisation and transformation of *E. coli* were performed as

described by Sambrook *et al.*, (1989). Analytical amounts of plasmid were isolated as

described by Birnboim (1983), whereas preparative amounts were purified with Qiagen

columns (Valencia, CA, USA). Total DNA from *B. cepacia* was isolated by sarcosyl-

pronase lysis as described by Better *et al.* (1983). Tri-parental matings from *E. coli* to *B.*

cepacia were performed with the helper strain *E. coli* (pRK2013) (Figurski & Helinski, 1979).

3.2.3   Identification of the *pehA* locus

Using the sequence data present in the databases, the *pehA* gene was amplified by PCR using two oligonucleotides (PEHAORF1, 5'-ATGAAAGGCAAAAGCAGCAC-3'; PEHAORF2, 5'-TCAAATCTGCGCAGGGATCG-3'). PCR amplification was performed in 50 µl volume, containing primers (0.5 µM each) deoxynucleotide triphosphates (200 µM each), chromosomal DNA (100 ng), and Vent DNA polymerase (0.5 U) in the buffer recommended by the manufacturer (New England Biolabs). After an initial incubation at 95°C for 5 min, 30 rounds of temperature cycling were performed under the following conditions: 95°C for 60 s, 55°C for 60 s, and 72°C for 60 s. The amplified fragment was cloned as a 1377 bp fragment in pGEM-T vector yielding pGEM-PEHA. This fragment was then used as probe against the cosmid library of *B. cepacia* ATCC 25416. A cosmid, designated pCOSPEHA was identified and the *pehA* gene was localised within a 2.5 kb *EcoRI-BamHI* fragment, which was cloned in the corresponding sites in pBluescript KS, yielding pBSPEHA.

3.2.4   Construction of a *B. cepacia* CepR based HSL detecting plasmid.

The *cep* genes of *B. cepacia* ATCC 25416 were used to construct a plasmid detecting HSL molecules. A *BamHI-HindIII* DNA fragment of 3.5 kb from pBIR containing the

whole *cepR* gene and the *cepI* promoter together with the first 120 bp of *cepI* were

inserted into the corresponding sites of the vector pQF50 (Farinha & Kropinski, 1990)

yielding pSCR1. Plasmid pQF50 contains a promoterless *lacZ* gene and in plasmid

pSCR1 the transcription of the *lacZ* gene is under the control of the *cepI* promoter.

For quantification of CepR activity, overnight *E. coli* DH5α (pSCR1) cultures were

normalised to an O.D. 600 nm of 0.1 in a volume of 20 ml LB containing the desired

HSL at the desired concentration. Cultures were then grown with agitation at 37°C for

six hrs and β-galactosidase activities were determined. Presence of HSL was also

detected on solid media by growing *E. coli* (pSCR1) on X-gal and ampicillin media in

close proximity to the tester strain. Presence of HSL was observed when *E. coli*

(pSCR1) turned blue.

3.2.5 Construction of a *B. cepacia* CepR based plasmid for the detection of DNA fragments controlled by quorum sensing.

The *B. cepacia* ATCC 25416 *cepR* ORF together with its promoter region were used to

construct a plasmid for the detection of DNA fragments containing promoters controlled

by quorum sensing. A 1347 bp DNA fragment from pBIR, containing the whole *cepR*

gene and its promoter, was amplified in a PCR reaction using two oligonucleotides

(CEPRD2, 5'-CTCCATGGGTAACGGTTTCTTGATCAAC-3'; CEPR-RA, 5'-TGGC

ATGCCCTCGTTCGAGGTCAGGGCG-3'). PCR amplification was performed in 50

μl volume, containing primers (0.5 μM each) deoxynucleotide triphosphates (200 μM

each), pBIR DNA (10 ng), and Taq DNA polymerase (0.5 U) in the buffer

recommended by the manufacturer (Promega). After an initial incubation at 98°C for 5

min, 35 rounds of temperature cycling were performed under the following conditions: 95°C for 30 s, 59°C for 60 s, and 72°C for 90 s. The amplified fragment was first cloned into pGEM-T vector, thus yielding pGEM-CEPR, following the instruction provided by the supplier (Promega). Consequently, it was digested and cloned from pGEM-CEPR as a *Nco*I-*Sph*I fragment into the corresponding sites of pQF50, yielding the master cloning vector pSCR2 (Figure 3).

The vector was first tested for its ability to detect quorum sensing-controlled fragments of DNA. This was done by amplifying a DNA fragment containing the promoter region of *cepI*, a quorum sensing-regulated gene (see Chapter 2; Lewenza & Sokol, 2001). A 250 bp DNA fragment from pBIR, containing the *cepI lux*-box and promoter elements, was amplified in a PCR reaction using two oligonucleotides (CEPID, 5'-GGTCGCGCTCGAAGCTTTCGTTCGCC-3'; CEPIR, 5'-CCCCGCGGATCCACGTC CTGATCGGCGTCA-3'). PCR amplification was performed in 50 µl volume, containing primers (0.5 µM each) deoxynucleotide triphosphates (200 µM each), pBIR DNA (10 ng), and Taq DNA polymerase (0.5 U) in the buffer recommended by the manufacturer (Promega). After an initial incubation at 98°C for 5 min, 10 rounds of temperature cycling were performed under the following conditions: 95°C for 30 s, 50°C for 30 s, and 72°C for 60 s; then 25 rounds of temperature cycling under the following conditions: 95°C for 30 s, 54°C for 30 s, and 72°C for 60 s. The amplified fragment was first cloned into pGEM-T vector, thus yielding pGEM-CEPI, following the instructions provided by the supplier (Promega). The fragment was sequenced to verify the fidelity of the PCR reaction. Consequently, it was digested and cloned from pGEM-CEPI as a *Bam*HI-*Hind*III fragment into the corresponding sites of either pSCR2

or pQF50, thus creating pSCR2C and pSCon used as positive and negative control, respectively.

Several unique restriction enzyme sites were present upstream of the *lacZ* gene in pSCR2 (Figure 3); these sites were used to clone fragments of genomic DNA of *B. cepacia* ATCC 25416, digested with different restriction enzymes. The enzymes used were *Nru*I, *Hae*III, *Hinc*II, *Alu*I and *Sau3A*I (all enzymes were purchased from New England Biolabs); single and double digestions were made with all of them. DNA ligations between pSCR2 and the genomic DNA fragments from *B. cepacia* were transformed in *E. coli* DH5α and plated on selective media containing 100 µg /ml ampicillin, X-Gal and 100 nM C_8-HSL. Blue colonies were identified and used in cross-streak experiments on X-gal/ampicillin plates, in close proximity to *B. cepacia*. Clones that turned blue only in proximity to *B. cepacia* were selected for further analysis.

3.2.6   Reporter gene fusion assay.

β-galactosidase activity was determined as described by Miller (1972) with the modifications of Stachel *et al.* (1985).

3.2.7   Exoenzyme assays, siderophore production and *in vitro* maceration of onion tissue.

For the determination of proteolytic and chitinolytic activity, strains were streaked on LB plates supplemented with 2 % skim milk powder (Difco, Detroit, USA) or 0.037% ethylene glycol chitin and 0.01 % trypan blue (both obtained from Sigma, Steinheim,

Germany), respectively. Lipolytic activity was determined on plates containing tributyrin agar base and 1 % glycerol tributyrate (both obtained from Sigma, Steinheim, Germany). Plates were incubated for 48 hours at 30 °C. The production of the relevant exoenzyme was indicated by the presence of zones of clearing surrounding bacterial growth due to substrate degradation. Siderophore activity was determined using Chromazurol S (CAS). On CAS agar plates, siderophores remove iron from the CAS dye complex, resulting in a blue-to-orange colour change in zones surrounding the colonies.

For the onion maceration experiments, clean, disease-free onions (*Allium cepa*) were wiped with 90% v/v alcohol and chopped aseptically in slices. Onion slices were placed into sterile Petri dishes and nicks of approximately 2 mm made in the tissue surface (Wigley & Burton, 1999). Overnight LB cultures of *B. cepacia* were normalized at O.D. 600 nm of 1 and 100 µl were inoculated onto the surface of onion tissue prepared as described. Plates were incubated at 30 °C, and readings were taken after 48 h of incubation.

For polygalacturonase (PehA) activity, bacteria was grown overnight in M9GP media with 0.2 % polygalacturonic acid (Gonzalez, *et al.*, 1997). Supernatants were concentrated 15 fold and dialyzed against 20 mM sodium acetate buffer at pH 5. For the determination of PehA activity, the following protocol was adapted from Schejter & Marcus (1988): enzymatic reaction was initiated by mixing an aliquot of the enzyme (100 µl) with 100 µl of sodium polypectate solution (0.05 % sodium polypectate, in 10 mM acetate buffer pH 4,2), and incubated at 30 °C. Then, 500 µl of Bernfeld reagent (50 mM 3,5-dinitro-salysilic acid (Sigma, Steinheim, Germany), 0.5 N NaOH (Merck,

Darmstadt, Germany), 1 M sodium potassium tartrate (CarloErba, Milano, Italy)) were added to the mix and the samples were heated in boiling water for 10 min. Absorbance was measured at 530 nm. When necessary, dilutions were made to be in the linear range of absortion (0.1-1). The unit of activity is the amount of enzyme that releases 1 μmol of reducing groups/min at 30 °C.

3.2.8 DNA sequence determination and analysis.

Nucleotide sequences were determined by the dideoxy chain-termination method (Sanger, *et al.*, 1977) using [^{35}S]-dATP for labelling and 7-deaza-dGTP (Pharmacia) instead of dGTP. Analysis of DNA sequence was performed with DNA Strider (CEA, France) or with EditSeq and MapDraw software (DNASTAR, Inc.). BLASTX and BLASTN programs were used to search the non-redundant sequence database for homologous sequences (Madden, *et al.*, 1996).

3.2.9 RNA assays.

Total RNA was extracted from either 2.5 ml of mid log *B. cepacia* growing cells (OD 600nm 3) or from overnight cultures, using the RNeasy Mini Kit according to the instructions provided by the supplier (Qiagen, Hilden, Germany). RNA concentration was estimated spectrophotometrically.

For primer extension analysis, about 25 μg of total RNA were annealed in hybridisation buffer (150 mM KCl, 10 mM Tris-Cl pH 8.0, 1 mM EDTA) with an [γ-^{32}P]-end-

labelled oligonucleotide (PEHA2: 5'-AACCTATGAGTCGCAATTCGCCGCC-3') for

90 min at 65 °C. Extension was performed by adding the reaction mix (30 mM Tris-Cl

pH 8, 15 mM MgCl2, 8 mM DTT, 250 µM dNTPs and 2 U AMV reverse transcriptase

(Promega)) and incubating for 1h at 42 °C. After this incubation period, samples were

treated with RNAse (2 µg RNAse A), extracted with phenol/choloform/isoamyl alcohol,

and further precipitated with ethanol. Pellets were then resuspended in 5 µl stop/loading

dye and fractionated on a 8 % polyacrylamide denaturing gel containing 7M urea. A

DNA sequence ladder was obtained with the M13 specific primer and pBluescript KS as

template, using $[\alpha\text{-}^{35}S]$ dATP for labelling and 7-deaza-dGTP (Pharmacia) instead of

dGTP, following the instructions provided by the supplier.

For Northern blot analyses, RNA (10 µg) of each sample was applied to a 2.2 M

formaldehyde 1,5 % agarose gel and electrophoresed according to (Sambrook, *et al.*,

1989). Transfer of the RNA to Hybond-N+ membranes (Amersham Biosciences),

prehybridisation, hybridisation, and washing of the filter were performed according to

Sambrook, *et al* (1989). Probes were radiolabelled with $[\gamma\text{-}^{32}P]$-dCTP, using a random

primed kit (Boehringer Mannheim).

3.2.10 Plasmids used.

Table 1. Plasmids used in this study.

Plasmid	Description[a]	Source or refrence
pBluescript KS	Ap[r]; ColE1 replicon	Stratagene
pUC18	Ap[r]; ColE1 replicon	(Yanisch-Perron, *et al.*, 1985)
pMP190	Cm[r]; IncQ; promoter probe vector	(Spaink, *et al.*, 1987)
pMP77	Cm[r]; IncQ; promoter probe vector	(Spaink, *et al.*, 1987)
pRK2013	Km[r]; Tra[+]; Mob[+]; ColE1 replicon	(Figurski & Helinski, 1979)
pPH1JI	Gm[r]; IncP1	(Beringer, *et al.*, 1978)
pLAFR3	Tc[r]; Broad-host-range cloning vector; IncP1	(Staskawicz, *et al.*, 1987)
pQF50	Ap[r]; Broad-host-range vector; pRO1600 replicon	(Farinha & Kropinski, 1990)
pBIR	Ap[r]; pBluescript KS containing *cepIR* locus in a 9 Kbp *EcoR*I fragment	This study
pMPIR	Cm[r]; pMP77 containing the *cepIR* locus in a 4.5 Kbp *Pst*I fragment	This study
pGEM-PEHA	Ap[r]; pGEM-T vector containing *pehA* as a 1377 bp fragment	This study
pCOSPEHA	Tc[r]; pLAFR3 containing the *pehA* locus	This study
pBSPEHA	Ap[r]; pBluescript containing the *pehA* locus in a 2,5 Kbp *EcoR*I-*BamH*I fragment	This study
pGEM-CEPR	Ap[r]; pGEM-T vector containing *cepR* as a 1347 bp *Nco*I-*Sph*I fragment	This study
pGEM-CEPR2	Ap[r]; pGEM-T vector containing P*cepI* as a 250 bp *BamH*I-*Hind*III fragment	This study
pSCR1	Ap[r]; pQF50 containing P*cepI*-*lacZ* and *cepR*	This study
pSCR2	Ap[r]; pQF50 containing *cepR* and polylinker	This study
pSCR2C	Ap[r]; pQF50 containing *cepR* and P*cepI*, used as positive control plasmid	This study
pSCon	Ap[r]; pQF50 containing P*cepI*, used as negative control plasmid	This study

[a] Ap[r], Cm[r], Gm[r], Km[r], Tc[r]: resistant to ampicillin, chloramphenicol, gentamycin, kanamycin and tetracycline, respectively.

3.3   RESULTS

3.3.1   Phenotypic characterisation of *B. cepacia* quorum sensing mutants.

The expression of several genes involved in the production of virulence factors, as well as other phenotypes, has been characterised for their dependence on expression based on an active quorum sensing system; examples can be found among several Gram-negative bacteria as *P. aeruginosa* (Brint & Ohman, 1995; de Kievit, *et al.*, 2002; Latifi, *et al.*, 1995; Pesci *et al.*, 1997), *C. violaceum* (Chernin, *et al.*, 1998; McClean, *et al.*, 1997), *Yersinia spp* (Atkinson *et al.*, 1999; Throup *et al.*, 1995), *Aeromonas* spp (Swift, *et al.*, 1997) or *Serratia liquefaciens* (Eberl, *et al.*, 1996).

In the present work, the production of several extracellular proteins and compounds were tested in *B. cepacia* ATCC 25416 and in the respective quorum sensing *cepI* and *cepR* null-mutant derivatives (Table 2). It was observed that 25416-I displayed similar lipolytic and chitinolytic activity when compared to the wild-type parent strain, whereas the proteolytic activity was significantly lower in the quorum sensing mutants; this observation was similar to what was observed in two different *B. cepacia* genomovar III quorum sensing mutants (Huber *et al.*, 2001; Lewenza *et al.*, 1999). The production of siderophores (iron transport compounds) was assayed on CAS indicator plates and it was observed that there was no alteration in the production of siderophores by the quorum sensing mutants when compared to the wild type parent strain. Interestingly, the two *B. cepacia* genomovar III quorum sensing mutants have been previously characterised with either an increase or decrease in siderophore production (Huber, *et*

al., 2001; Lewenza, *et al.*, 1999). Finally, quorum sensing did not play a role in swimming or swarming motility in *B. cepacia* ATCC 25416, again in contrast with the results obtained with other members of the *B. cepacia* complex (Huber, *et al.*, 2001). Table 2 summarises these results and compares them to the observations reported in two other *B. cepacia* strains with respect to quorum sensing.

Table 2. Phenotypic characterization of *B. cepacia* ATCC 25416 and cep-defective mutant: comparison with *B. cepacia* H111 and *B. cepacia* K56-2[a].

Phenotype	*B. cepacia* ATCC 25416 (Genomovar I)		*B. cepacia* H111 (Genomovar III)		*B. cepacia* K56-2 (Genomovar III)	
	WT	25416-I	WT	H111-I	WT	K56-2I
HSL	+	-	+	-	+	-
Protease	+	-	+	-	+	-
Lipase	+	+	+	+	+	+
Chitinase	+	+	+	+/-	+	n.d
Swimming	+	+	+	+	n.d.	n.d.
Swarming	+	+	+	-	n.d.	n.d.
Siderophore	+	+	+	+/-	+	++
Onion maceration	+	+/-	n.d.	n.d.	n.d.	n.d.

[a] Production of HSL, synthesis of extracellular enzymes, production of siderophore were all tested as described in Materials and Methods. The experiments were done in triplicate, obtaining the same results for *B. cepacia* 25416-I and *B. cepacia* 25416-R (with the only exception of HSL production, discussed in Chapter 2). For simplification, only the results corresponding to 25416-I are shown. The data presented for *B. cepacia* H111 and *B. cepacia* K56-2 are described in Huber *et al.*, (2001) and Lewenza *et al.*, (1999), respectively. +, activity exibited by the wild type; +/-, significantly reduced activity; ++ significantly increased activity; -, no detectable activity; n.d., not determined.

As depicted in Table 2 and Figure 1, the quorum sensing mutant shows an attenuated onion maceration activity when compared to the parent strain. This could be an indication that *B. cepacia* ATCC 25416 employs quorum sensing in order to exert its onion pathogenicity.

3.3.2   *pehA* expression is modulated by quorum sensing in *B. cepacia.*

As described above, it was observed that a quorum sensing mutant shows an attenuated onion maceration activity. Interestingly, when this quorum sensing mutant was complemented with the plasmid pMPIR carrying the *cepIR* locus, the maceration activity was apparently higher than the wild type strain (Figure 1).

In *B. cepacia* ATCC 25416, the *pehA* gene encodes for polygalacturonase (PehA), a secreted enzyme that has been suggested to be a virulence factor, important for the onion maceration activity of this strain (Gonzalez, *et al.*, 1997). It has been reported that the PehA activity can be induced in M9GP medium, which contains polygalacturonic acid, the substrate of the enzyme (Gonzalez, *et al.*, 1997). Therefore, it was of interest to investigate if the extracellular PehA activity would require quorum sensing, in addition to the inducer substrate, for its maximal expression. Consequently, PehA activity was determined from spent M9GP overnight culture supernatants (see Materials and Methods for details) of *B. cepacia* ATCC 25416 and *B. cepacia* 25416-I and, as can be seen in Table 3, a 40 % reduction in the specific activity was observed in the quorum sensing null-mutant *B. cepacia* 25416-I. The PehA activity was also determined from the spent culture supernatant of the complemented mutant 25416-I (pMPIR) and, as

depicted in Table 3, the activity was higher than the wild type parent strain, which is in

agreement with the observations in the onion maceration experiments (Figure 1). These

results strongly suggest that the production of PehA activity is regulated in *B. cepacia*,

at least in part, by quorum sensing via the CepIR system.

B. cepacia	*B. cepacia*	*B. cepacia*
ATCC 25416	25416-I	25416-I (pMPIR)
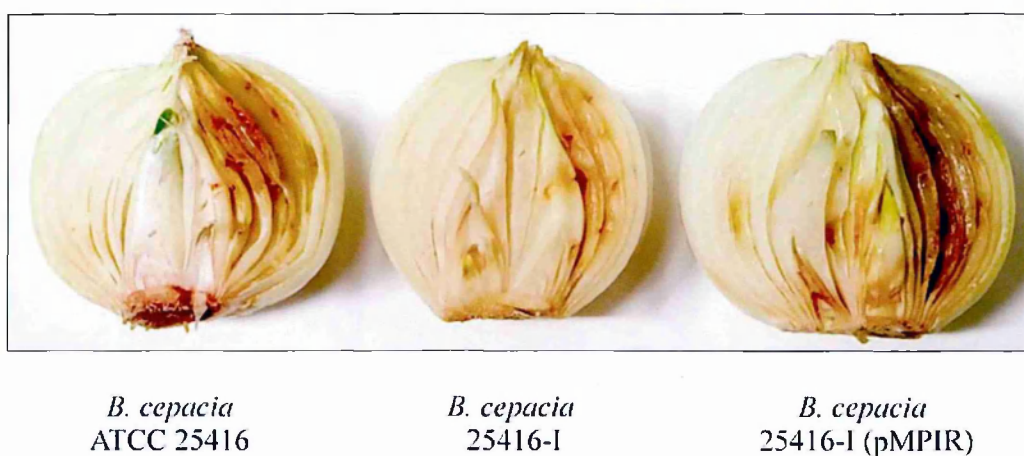
Figure 1. Role of quorum sensing in onion rot. Cultures of *B. cepacia* (100 μl of an
overnight culture of the indicated strain, normalised to an O.D. 600 nm of 1) were
inoculated on the right half of the onion, treated as described in Materials and Methods.
Onions were incubated at 30 °C for 48 hours. The picture is representative of the
experiment done in triplicate.

Table 3. Polygalacturonase activity from spent culture supernatants of *B. cepacia* ATCC 25416 and 25416-I.

B. cepacia strain	Polygalacturonase activity (U/mg)[a]	% of ATCC 25416 polygalacturonase activity
ATCC 25416	2300	100
25416-I	1380	60
25416-I (pMPIR)	3220	140

[a]Cultures were grown for 16 h in M9GP medium (Gonzalez, *et al.*, 1997) and had similar OD_{600}s. For growth media and enzyme activity assay, see Materials and Methods. The mean value of triplicate experiments are given, and standard deviations were +/-5%.

3.3.3   The polygalacturonase *pehA* promoter

Following the observation that the polygalacturonase activity was regulated by quorum sensing (see above), the *pehA* promoter was subjected to further molecular characterisation. The complete sequence of the *pehA* ORF has been deposited in the sequence databases (Gonzalez, *et al.*, 1997); however, no information is available about the upstream non-coding promoter region of the gene. For this reason, a clone from the genomic cosmid bank, designated pCOSPEHA, was identified by hybridisation with a DNA probe encoding the *pehA* gene. Further sub-cloning experiments yielded pBSPEHA, which harbours a 2.5 Kbp *Eco*RI-*Bam*HI, coding for the complete *pehA* locus. DNA sequencing of this fragment provided the necessary information in order to

continue with the characterisation of the *pehA* promoter. Synthetic oligonucleotides were designed in the putative promoter region of *pehA* and primer extension experiments were performed in order to localise the start site of transcription of *pehA*. As depicted in Figure 2a, a single extension product of 178 bp was obtained upstream from the *pehA* translation start codon. It was possible to identify putative -35, -10 and SD promoter elements (Figure 2b). In addition, it was also possible to identify a putative *lux*-box, partially overlapping the putative -35 promoter element (Figure 2b). The putative *lux*-box was highly similar to the *lux*-box of *cepI*, having 12 identical nucleotides out of 20 (Figure 2c).

(a)

(b)

```
CGCCATTGTGATTGCTGCTCGCACTCCCGTCAGTGCCATCACTTTGCCAACGTTCTCAACTGAGGA
                         putative lux-box        -35

TCATTATGGTCACCGATCCGTGTTAGTGCGCAGGGATTCGAGAAAAATCCGAGGCCCTGAGCAGTA
  -10           +1

TCCTGGGATACGTGTTGTG...89bp...TTAAATACAAGGAAAACCAATGAAAGGCAAAAGCAGC
                                        S.D
```

(c)   *cepI*   CCCTGTAAGAGTTACCAGTT

                          ** ** ** *   * ** **

            pehA   TCCCGTCAGTGCCATCACTT
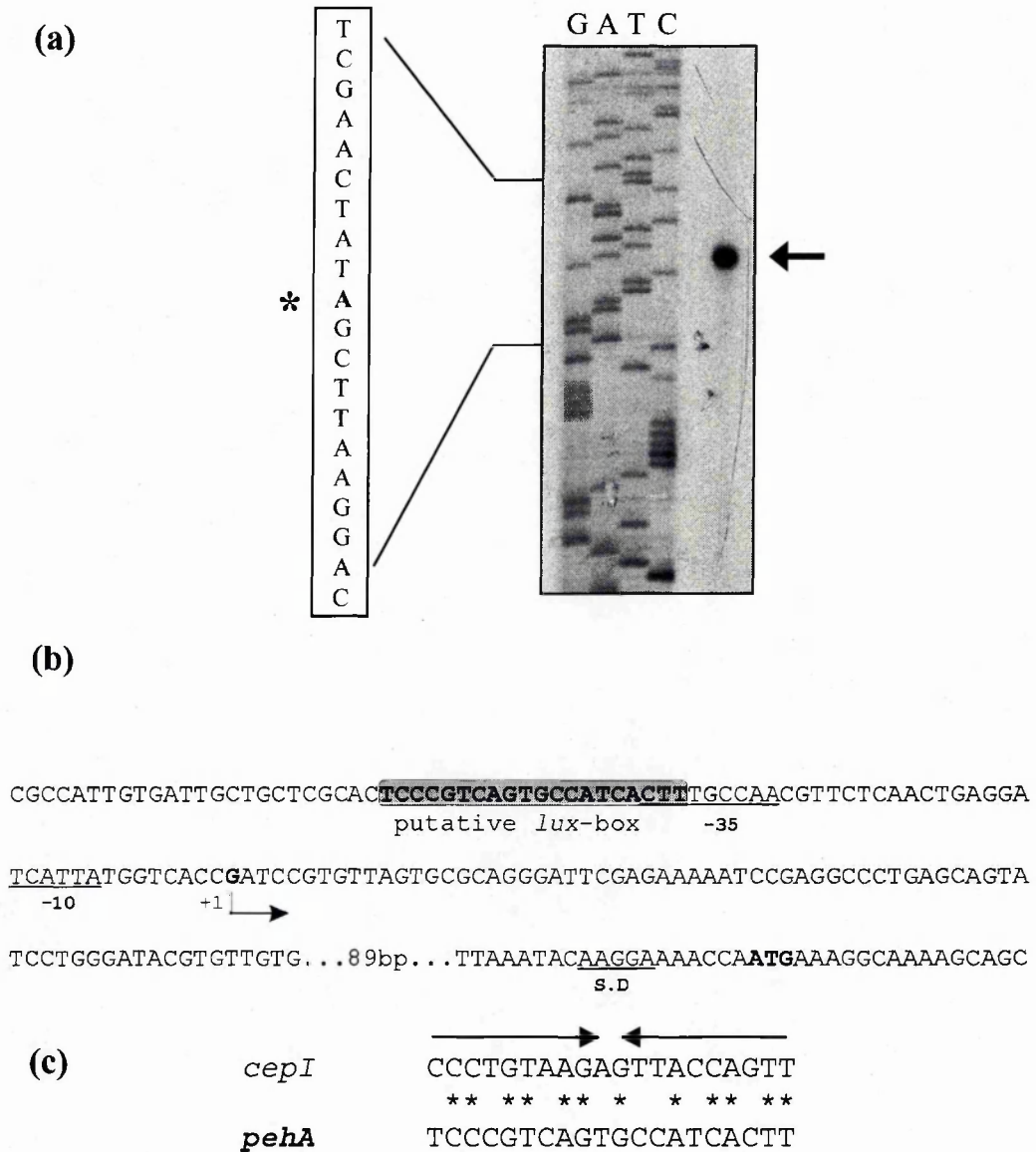
Figure 2. The *pehA* promoter analysis. (a) The arrow indicates the primer extension product, visible as a single band. At the left side, pBluescript KS sequence is shown; evidenced in bold and with an asterisk is the nucleotide from which the initiation of transcription was deduced. (b) Putative -10 and -35 sites, the ATG translation start codon, and the Shine-Dalgarno sequence (S.D.) are depicted. The arrow indicates the deduced start of transcription. (c) Comparison between the *pehA* putative *lux*-box and the *cepI lux*-box. Identical nucleotides are indicated by an asterisk.

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3.3.4   Identification of quorum sensing-regulated genes in *B. cepacia*

It was of interest to perform a systematic investigation of global gene regulation by quorum sensing in *B. cepacia*. In order to do this, it was necessary to develop a methodology for the identification of quorum sensing-regulated loci in *B. cepacia*. To date, no report is available regarding the molecular identification and characterisation of the quorum sensing regulon in *B. cepacia*. The cloning vector pSCR2 was constructed and, as depicted in Figure 3, it contains the *cepR* gene and a promoterless *lacZ* gene preceded by a series a unique enzyme restriction sites. The experimental strategy of the series of following experiments was to clone fragments of genomic DNA of *B. cepacia* in pSCR2, selecting for those that were able to activate transcription in the presence of exogenously added C_8-HSL in *E. coli* DH5α (complete experimental strategy is depicted in Figure 4). This activation was evidenced by the presence of blue colonies on selective plates containing ampicillin, X-Gal and 100 nM C_8-HSL. A second step was necessary in order to discriminate those colonies in which transcription was constitutively activated irrespective of the presence of C_8-HSL. This necessary discrimination was overcome by cross-streaking the blue colonies obtained in the previous step in close proximity with *B. cepacia* ATCC 25416 in the absence of any exogenously added C_8-HSL.
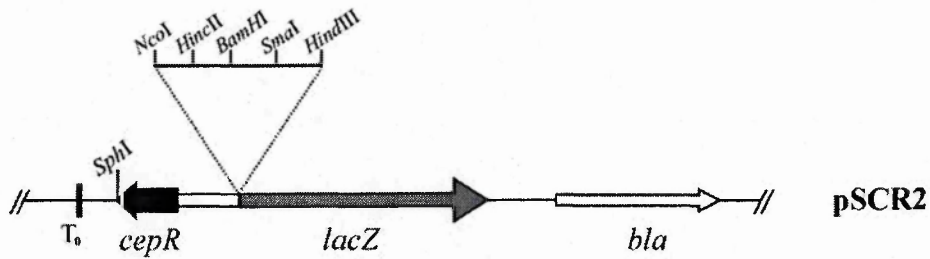
Figure 3. Schematic diagram (not to scale) of the *cepR*-based cloning plasmid used to identify quorum sensing-regulated genes. Plasmid pSCR2 contains the *cepR* gene cloned in the broad-host-range plasmid pQF50. The genetic components are: *cepR*, gene encoding the transcriptional activator CepR; *lacZ*, promoterless β-galactosidase gene; T_0, transcriptional *trpA* terminator; bla, ampicillin-resistance marker. The indicated restriction sites in the polylinker were used to clone DNA fragments obtained from genomic DNA of *B. cepacia* ATCC 25416, digested with compatible-ends restriction enzymes (see Materials and Methods for details). Positive clones were identified as blue colonies on selective plates in the presence of C_8-HSL.

Those colonies that turned blue only in proximity to *B. cepacia* were postulated to need the HSLs molecules in order to activate the promoter. This was further confirmed since these *E. coli* colonies harbouring *B. cepacia* DNA fragments in pSCR2 were not activated by the *cepI* null mutant 25416-I when cross-streaked in close proximity (see below). Around 50,000 clones were obtained, using various enzyme restriction sites, in the first step of the experiment and from those, between 4-5 % (approx. 2300) were blue on selective plates containing X-gal and C_8-HSL. Of these clones, the second step revealed that approximately 200 clones were positive when cross-streaked in proximity
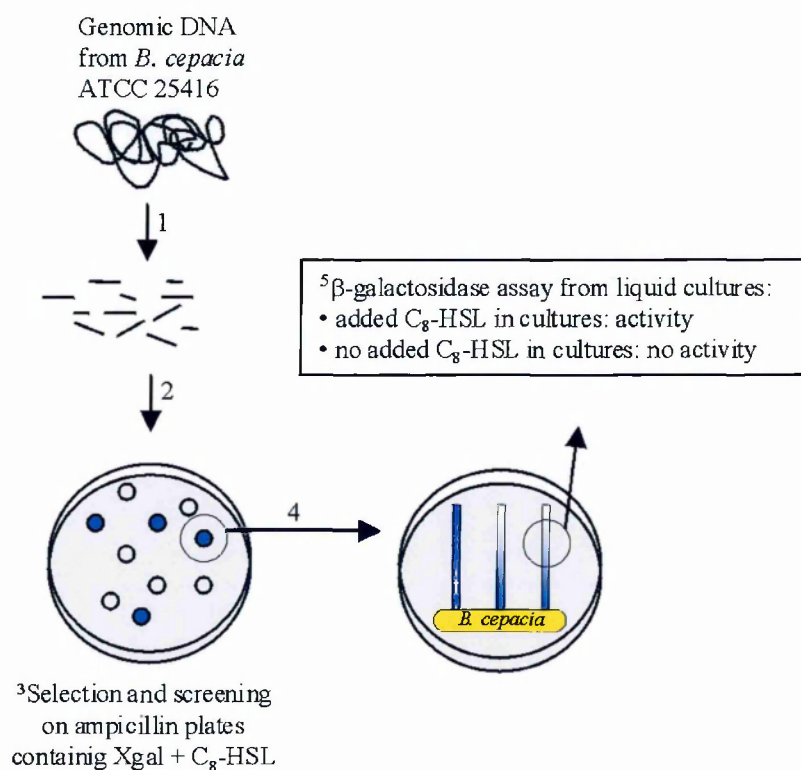
Figure 4. Schematic diagram of the strategy used to identify quorum sensing-regulated genes. *B. cepacia* ATCC 25416 genomic DNA was digested with different restriction enzymes [1](*Nru*I, *Hae*III, *Hinc*II, *Alu*I, *Sau3A*I), cloned in the compatible sites of the vector pSCR2 [2](*Nco*I, *Hinc*II, *Bam*HI, *Sma*I, *Hind*III) transformed in *E. coli* DH5α and spread on selective plates containing ampicillin, Xgal and 100nM C_8-HSL [3](approximately 50000 colonies were obtained). Then, blue colonies [4](approximately 2300) were subjected to T-streak analysis and those blue just in proximity of *B. cepacia* were analysed using a β-galactosidase assay from liquid cultures to confirm the C_8-HSL dependency of expression [5](30 out of 200 had activity only in presence of C_8-HSL).

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to *B. cepacia* ATCC 25416, i.e. were blue only in close proximity to the strain. This group of clones was further analysed to determine the strength of the transcriptional activation, using β-galactosidase assays from liquid cultures in the presence and absence of 100 nM of C_8-HSL in the liquid media. Finally, it was established that 30 clones (representing approximately 0,1 % of the total number of clones screened) showed activation of transcription in *E. coli* in a [CepR-C_8-HSL]-dependent manner, since when C_8-HSL was included in the liquid media, the β-galactosidase activity was 5 to 25 fold higher than when C_8-HSL was not included (Table 4). Further evidence confirming this observation is presented in Figure 5, where some clones listed on Table 4 are subjected to a cross-streak with either *B. cepacia* ATCC 25416 or *B. cepacia* 25416-I, showing the [CepR-C_8-HSL]-dependent expression of the reporter gene.

P67		ᵗ P121	P67		P121
P80		P88	P80		P88
P114		P94	P114		P94
P53		P110	P53		P110
pSCon		pSCR2C	pSCon		pSCR2C
P91		P85	P91		P85
P96		P55	P96		P55
P111		P79	P111		P79

B. cepacia ATCC 25146   *B. cepacia* 25146-I
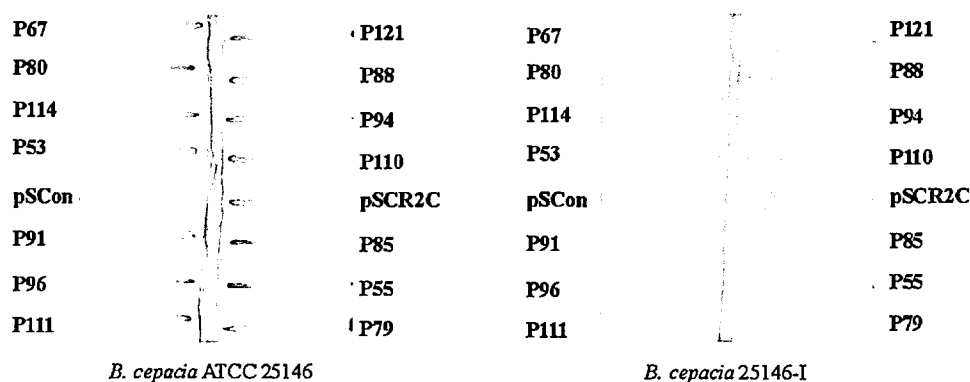
Figure 5. Cross-streak experiments. Some of the clones listed in Table 4 were cross-streaked in close proximity to either *B. cepacia* ATCC 25416 or *B. cepacia* 25416-I in plates containing 100 μg/ml ampicillin and X-Gal. Blue colour is developed only in proximity to the WT strain, thus further confirming the [CepR-C_8-HSL]-dependent expression of the clones. pSCR2C and pSCon, positive and negative control, respectively.

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3.3.5 DNA sequence analysis of the identified clones which contained putative quorum sensing regulated promoters.

The DNA sequence of each fragment was determined and compared to the sequence data (not yet annotated) of the genome project of *Burkholderia cepacia* J2315, a genomovar III strain of the *B. cepacia* complex (http://www.sanger.ac.uk/Projects/B_cepacia/), using the BLASTN program. In the majority of the cases, the clones obtained here displayed DNA identity with a region of the genome of *B. cepacia* J2315 grater than 80%, and those having a percentage well below to this value, usually very short fragments, were not considered for further analysis. The *B. cepacia* J2315 DNA, flanking 2 kbp upstream and 2 kbp downstream of the highly identical sequence of each clone, was consequently used in the ORF finder program (http://www.ncbi.nlm.nih.gov/gorf/), followed by a BLASTP analysis. In this way, a complete picture of the putative ORFs present or just downstream to the putative promoter sequence of each clone was obtained. In this *in-silico* analysis it is postulated that a considerable homology and a similar gene arrangement exists between *B. cepacia* ATCC 25416 and *B. cepacia* J2315 so as to predict possible gene(s) which are regulated by the putative quorum sensing regulated promoters identified here. As depicted in Table 4, within the sequence of some of the identified clones, it was possible to identify ORFs that encode for homologues of known proteins; however, not all the clones have sequences from which an ORF can be deduced. Through this analysis, it was possible to identify some homologues of proteins from different organisms believed to be virulence factors as for example PpiaseB (P91), Malate synthase (P67) or PilA (P15).

Table 4. Quorum sensing-controlled genes in *Burkholderia cepacia* ATCC 25416.

Clone	Enzyme[a]	Quorum sensing-regulated ORF	Identity[b] (%)	Activity[c] C₈HSL (+)	Activity[c] C₈HSL (-)
67	N	malate synthase A from *R. solanacearum*	79	28	1
80	N	unknown		27	1
114	N	hypothetical protein from *B. fungorum*	72	26	1
53	N	AidA from *R. solanacearum*	56	17	2
91	A-Hi	peptidyl-prolyl cis-trans isomerase PpiaseB from *R. solanacearum*	80	24	1
96	A-N	hypothetical protein from *B. fungorum*	40	24	1
111	Hi-N	hypothetical protein from *B. fungorum*	56	25	2
121	Hi-N	putative ribonucleoside reductase 1 from *R. solanacearum*	82	15	1
88	A-N	unknown		15	1
94	A-N	unknown		19	1
110	Hi-N	putative amino acid transport signal peptide protein from *R. solanacearum*	70	15	1
85	A-N	unknown		17	1
55	N	sugar-binding periplasmic signal peptide protein from *R. solanacearum*	89	11	1
79	Ha	hypothetical protein from *Ralstonia metallidurans*	34	16	1
69	N	ExbB from *Bordetella bronchiseptica*	38	9	1
103	A-Hi	catechol 1,2-dioxygenase from *Burkholderia sp.* TH2	73	13	1
81	A	probable penicillin-binding 3 precursor PBP-3 transmembrane protein from *R. solanacearum.*	66	15	1
56	N	acetaldehyde dehydrogenase II from *Alcaligenes eutrophus*	73	7	1
57	S	probable porin transmembrane protein from *R solanacearum*	41	10	1
105	A-N	hypothetical protein from *R. solanacearum*	61	12	1
59	S	unknown		6	1
68	N	hypothetical protein from *B. fungorum*	77	10	1
130	Hi-N	unknown		9	1
25	N	putative ribonucleoside reductase from *R. solanacearum*	76	7	1
122	Hi-N	unknown		8	2
135	A-Hi	hypothetical protein from *B. fungorum*	30	9	1
15	Hi	PilA pilus from *Caulobacter crescentus* CB15	53	6	1
38	A	NADH-flavin reductase from *Vibrio vulnificus* CMCP6	41	5	1
1	A	unknown		6	1

[a]Restriction enzymes used to digest *B. cepacia* ATCC 25416 genomic DNA, N: *Nru*I; Ha: *Hae*III; Hi: *Hinc*II, A: *Alu*I and S: *Sau3A*I.
[b]Amino acid identity to the complete ORF obtained from the *B. cepacia* J2315 sequence database. For some of them, it was not possible to find identity to any ORF from the databases and those are showed as unknown.
[c]Activity expressed in Miller units, obtained from overnight cultures in presence (+) or absence (-) of C₈-HSL.

Other homologues were metabolic proteins as for example NADH-flavin reductase (P38), catechol 1,2 dioxygenase (P103) or ribonucleoside reductase (P25) and also membrane-associated proteins as a porin transmembrane protein (P57), or ExbB (P69). Interestingly, a homologue of AidA (autoinducer dependent) was also identified (P53). This protein of unknown function has been previously described for its quorum sensing dependent expression in *Ralstonia solanacearum* (Flavier, *et al.*, 1997b). A preliminary characterisation of the expression of the corresponding gene *aidA* was made for *B. cepacia* ATCC 25416 in the present study (see below). Some of the identified clones from Table 4 that displayed higher levels of β-galactosidase activity were selected and were further characterised by *in silico* analysis. This was done by identifying the putative ORFs surrounding the sequences of P67, P80, P114, P53, P91 and P111. Using the BLASTP program, an approximate annotation of the region surrounding 2 kbp to each sequence was made. The results of this analysis are depicted in Figure 6 and, as expected, it appears that the promoter regions of several ORFs were identified for the different clones subjected to the analysis described above. However, it must be stressed that this analysis was made over the genome of *B. cepacia* J2315, a genomovar III member of the *B. cepacia* complex, making the assumption that the genes and the genetic arrangement of the genes will be comparable to what can be found in *B. cepacia* ATCC 25146. This assumption is especially important for some clones such as P80, in which the DNA fragment containing a putative promoter regulated by quorum sensing does not code for an obvious ORF in the sequence, but the putative ORF that may be regulated is downstream of the sequence obtained in the experiments.

P67

ORF 2   ORF 1

700 bp

96% DNA homology to *B. cepacia* J2315

ORF 1: 79% amino acid identity to malate synthase A from *Ralstonia eutropha*
ORF 2: 78% amino acid identity to a probable transcriptional regulator (lysR family) from *R. solanacearum*

P80

ORF 3 ORF1   ORF 2

440 bp

80% DNA homology to *B. cepacia* J2315

ORF 1: 92% amino acid identity to DpsA from *B. pseudomallei*
ORF 2: 85% amino acid identity to catalase-peroxidase KatG from *B. pseudomallei*
ORF 3: 52% amino acid identity to 4-hydroxybenzoate octaprenyl transferase from *Xanthomonas axonopodis* pv. *citri* str. *306*

P114

ORF 3   ORF   ORF 2

194 bp

93% DNA homology to *B. cepacia* J2315

ORF 1: 43% amino acid identity to NAD-glutamate dehydrogenase from *Neurospora crassa*
ORF 2: 36% amino acid identity to a probable transcriptional regulator from *Pseudomonas aeruginosa* PAO1
ORF 3: 51% amino acid identity to YraQ from *E. coli* K12

P53

ORF 1   ORF 2

644 bp

86% DNA homology to *B. cepacia* J2315

ORF 1: 56% amino acid identity to Aida from *R. solanacearum*
ORF 2: 54% amino acid identity to bactoprenol glucosyl transferase (GtrB) from *Shigella flexneri*

P91

ORF1   ORF 2   ORF 3

414 bp

96% DNA homology to *B. cepacia* J2315

ORF 1: 58% amino acid identity to Peptidyl prolyl cis-trans isomerase A (PpiaseA) from *R. solanacearum*
ORF 2: 80% amino acid identity to Peptidyl prolyl cis-trans isomerase B (Ppiase B) from *R. solanacearum*
ORF 3: 53% amino acid identity to UDP-2,3-diacylglucosamine hydrolase from *R. solanacearum*

P111

ORF1   ORF 2

513 bp

85% DNA homology to *B. cepacia* J2315

ORF 1: 34% amino acid identity to short-chain dehydrogenase PA1829 from *P. aeruginosa* PAO1
ORF 2: 68% amino acid identity to short-chain dehydrogenase-reductase from *P. putida* K2240

Figure 6. Putative genetic elements present in the flanking regions of the identified sequences. The DNA sequence present in the selected clones, was compared to the genome of *B. cepacia* J2315. *In silico* analysis, with flanking *B. cepacia* J2315 genomic DNA (2Kbp upstream and downstream, respectively) was made using the ORF finder program. The percentage of DNA homology between *B. cepacia* ATCC 25416 and J2315 for each clone is indicated, as well as the amino acid identity of the putative ORFs found in each case with sequences present in the non-redundant databases. The arrows represent the position and size of the DNA genomic fragment, in scale to the putative ORF found.

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3.3.6   P53 transcription is quorum sensing-dependent.

In order to start a more accurate characterisation of some of the identified putative promoters, Northern hybridisation was performed using *B. cepacia* ATCC 25416 and *B. cepacia* 25416-I total RNA, so as to determine any difference in the transcription profile due to an interruption in the quorum sensing system (see Materials and Methods for technical details). The DNA probe was generated by PCR using primers designed to amplify the region described in Figure 6 for clone P53. As depicted in Figure 7, it was possible to observe a clear difference in mRNA abundance, when comparing *B. cepacia* ATCC 25416 and *B. cepacia* 25416-I. This result further validated at the molecular level the above-described strategy aimed to identify quorum sensing-controlled genes.
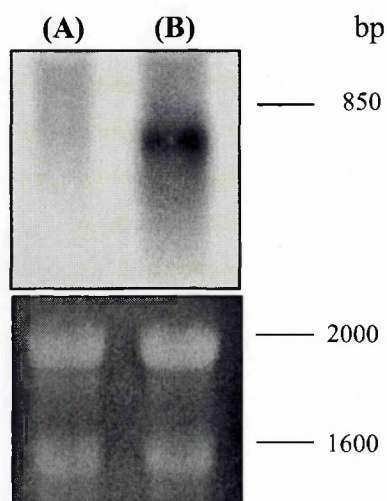
Figure 7. Effect of quorum sensing on P53 transcription. Northern blot hybridisation was performed using total RNA extracted from *B. cepacia* 25416-I (A) and *B. cepacia* ATCC 25416 (B); P53 DNA was radiolabelled and used as probe (see Materials and Methods). The lower panel is a loading control ethidium bromide-stained formaldehyde gel, showing that the amounts of rRNA were approximately equal.

3.4   DISCUSSION

In this chapter, the quorum sensing regulon of *B. cepacia* ATCC 25416 was further characterised. Several phenotypes were tested in order to assign a role to quorum sensing in their regulation. It was found that, in contrast to other members of the *B. cepacia* complex, quorum sensing in *B. cepacia* ATCC 25416 does not modulate the expression of chitinase, siderophores, swimming or swarming motility. On other hand, onion maceration experiments showed that quorum sensing is involved in the onion pathogenicity of *B. cepacia*. Moreover, the extra cellular protein polygalacturonase (PehA) has been previously described as an important determinant for the maceration of onions (Gonzalez *et al.*, 1997) and in this chapter it is shown that *B. cepacia* ATCC 25416 uses quorum sensing to positively modulate the expression of *pehA* in order to exert its pathogenicity. Finally, this chapter describes a series of experiments aimed to identify genes controlled by quorum sensing in *B. cepacia* ATCC 25416, using an HSL-sensor plasmid described in Chapter 2.

3.4.1   Investigating possible phenotypes regulated by quorum sensing in *B. cepacia* ATCC 25416.

The role of quorum sensing in the modulation of the expression of several phenotypes was analysed in *B. cepacia* ATCC 25416. As depicted in Table 2, while some of the tested phenotypes, in relation to quorum sensing, such as extra cellular protease and lipase production were similar to the results observed in previously described strains of *B. cepacia*, other results such as chitinase production and swarming motility were in

contrast. In *B. cepacia* H111 quorum sensing plays an important role in these two last phenotypes. This is evident since a reduction of the expression of both was observed when tested in the quorum sensing-deficient mutants (Huber, *et al.*, 2001). Moreover, it has also been demonstrated that swarming motility is important either for biofilm formation or for biofilm development at later stages in strain H111 (Huber, *et al.*, 2001; Huber, *et al.*, 2002). In the present study, no difference in expression in chitinase production or in swarming motility was observed in the *B. cepacia* ATCC 25416 quorum sensing mutants. Another contrasting result was obtained when the production of siderophores was tested. In *B. cepacia* K56-2 it has been reported that there is a significant rise in siderophore production when quorum sensing was interrupted (Lewenza, *et al.*, 1999; Lewenza & Sokol, 2001), in contrast, a significant reduction in siderophore production was observed in *B. cepacia* H111 in the *cep*-deficient mutants (Huber, *et al.*, 2001). In the present study, no difference in siderophore production was observed in *B. cepacia* 25416-I or *B. cepacia* 25416-R when compared to the wild-type parent strain. One possible explanation for these discrepancies can be attributed to the different strains used in these studies, and that they belong to different genomovars. These observations highlight the fact that, with the exception of protease production, there is no common phenotype known to be regulated by quorum sensing among different members of the *B. cepacia* complex.

3.4.2 Onion pathogenicity, expression of polygalacturonase (PehA) and the quorum sensing regulon.

B. cepacia ATCC 25416 was originally described as a phytopathogen, responsible for the rot of onions (Burkholder, 1950). The results obtained in onion maceration experiments presented in this study, strongly suggest that quorum sensing is at least in part participating in the modulation of this process, since the non-HSL producer strain *B. cepacia* 25416-I is less aggressive in onion maceration (Figure 1). Interestingly, it seems that the complemented *cepI* mutant not only restores its maceration ability, but it is more aggressive than the wild-type parent strain (Figure 1), a phenomenon that can be explained because of the multiple copies present of the plasmid inside the cell, possibly resulting in a stronger activation.

Another aspect that was covered in this chapter refers to the regulation of expression of the extra cellular enzyme polygalacturonase in *B. cepacia* ATCC 25416. The polygalacturonase activity is involved in the degradation of pectin, a complex heteropolysaccharide found in the middle lamella and the primary cell wall of higher plants and fruits (ten Have *et al.*, 1998). This enzyme is important in the mediation of enzymatic degradation of the plant cell wall during pathogenic attack by fungi or bacteria (Wagner *et al.*, 2000). Plant-pathogenic *B. cepacia* strains characteristically produce the extra cellular enzyme polygalacturonase (PehA), which is responsible for the onion maceration and is implicated in disease development (Ulrich, 1975).

It has been demonstrated by others that the PehA activity in *B. cepacia* can be induced in minimal media, when supplementing with polygalacturonic acid (Gonzalez, *et al.*, 1997). The data obtained in the present study revealed that the expression of the

polygalacturonase activity in M9GP media is positively modulated by quorum sensing

in *B. cepacia* ATCC 25416. As depicted on Table 3, the quorum sensing mutant

produces around 60 % of the extracellular PehA activity found in the wild-type parent

strain. In accordance with the observations made in the onion maceration experiments,

PehA activity was higher (140 %) in the complemented mutant 25416-I (pMPIR) when

compared to the wild-type strain. In summary, these results highly suggest that onion

pathogenicity in *B. cepacia* ATCC 25416 is primarily mediated by PehA, in agreement

with Gonzalez *et al.* (1997), and that the expression of the *pehA* gene, coding for this

enzymatic activity, is positively regulated by quorum sensing.

In order to further characterise the *pehA* locus, studies were performed to analyse the

putative promoter elements of this gene. The 5'end of the *pehA* mRNA was identified

by primer extension at 178 nucleotides upstream from the *pehA* translation start codon

(Figure 2a, 2b). It was possible to identify a putative S.D. ribosome binding sequence, -

10 and -35 promoter elements. The unusual long untranslated region of this promoter is

interesting, since it is possible that the regulation of the expression of the *pehA* gene can

be modulated at the post-transcriptional level. An example of such a type of regulation

is present in the *rpoS* gene of *E. coli*, where there is a long untranslated 5' region of 567

nucleotides that is subjected to a fine control of *rpoS* translation (Hengge-Aronis,

2002b).

In addition, it was also possible to identify a putative *lux*-box, known to be recognized

by LuxR family members, partially overlapping the putative -35 element (Figure 2b).

This overlapping feature is in agreement to what has been described in other organisms

(Egland & Greenberg, 1999; Finney, *et al.*, 2002). The putative *lux*-box of *pehA* was

compared to the *lux*-box of *cepI* and found to be highly similar, having 12 identical nucleotides out of 20 (Figure 2c). These observations highly suggest that the putative *lux*-box found in the *pehA* promoter could be a functional quorum sensing-regulatory element in *B. cepacia* ATCC 25416. To verify this hypothesis, a possible experiment that can be done is to make mutations in this region, expecting a dramatic reduction in the *pehA* expression, alternatively, DNA binding studies between CepR and this promoter could also reveal if this *lux* box if functional

3.4.3   Identifying genes controlled by quorum sensing in *B. cepacia* ATCC 25416

The last part of this chapter describes a technique aimed to identify genes controlled by quorum sensing in *B. cepacia*. A cloning vector (pSCR2) was constructed for the detection of genomic DNA fragments that activate transcription of a reporter gene in a [CepR-HSL]-dependent way, in the *E. coli* background. It was found that 30 independent DNA fragments most probably contained promoters that were able to activate transcription in a [CepR-C_8-HSL]-dependent way as concluded from β-galactosidase assays from liquid cultures (Table 4) and from cross-streak on solid media experiments (Figure 5). Noteworthy, when CepR is absent from the system (pSCon, Figure 5a), or when an HSL is not externally provided (Table 4 and Figure 5b), there is no significant activation of transcription, thus suggesting that the *E. coli* background is appropriate for the investigations shown here, likely not altering the results obtained.

The selected clones are depicted in Table 4 and, as can be seen, for some of them it was possible to identify a putative ORF present in the sequence of the genomic fragment

downstream of a putative promoter element. This was the case, for example, of clone P67, in which part of the sequence encodes for a polypetide that shares 75% amino acid identity to malate synthase from *R. solanacearum*. The malate synthase enzyme belongs to the glyoxylate cycle, which has been associated with virulence in organisms as *M. tuberculosis* or *C. albicans* (Lorenz & Fink, 2001; McKinney *et al.*, 2000). For P91, it was found an ORF encoding a polypeitide possessing 71% amino acid identity with PPiase (peptidyl-prolyl cis/trans isomerase) from *R. solanacearum*. An example of a protein with PPiase activity is Mip (macrophage infectivity potentiator) from *Legionella pneumophila*, which has been described as an essential virulence factor, important for invasion and intracellular establishment of this organism in macrophages and protozoa (Fischer *et al.*, 1992; Ludwig *et al.*, 1994). Interestingly, it was found that clone P15 encodes for a PilA homologue, a protein required for virulence and twitching motility in *P. aeruginosa* (Glessner, *et al.*, 1999) and *R. solanacearum* (Liu *et al.*, 2001). However, current evidence indicates that quorum sensing is not required for the expression of *pilA* in both *P. aeruginosa* and *R. solanacearum*, but rather is required for the assembly of functional pili (Kang *et al.*, 2002). Since in the present study the expression of this gene was found to be [CepR-C_8-HSL]-dependent, it seems that the regulation could be different in *B. cepacia*.

Another interesting locus that was found during the present study was that of clone P69, which encodes for a homologoue of ExbB, a member of the cytoplasmic membrane complex TonB, ExbB and ExbD that is involved in the transport of iron siderophores, haem/haemin, transferrin and vitamin B_{12} in various Gram-negative bacteria (Nicholson & Beall, 1999). On the other hand, within the sequence of clone P53 there is an ORF

that is 56% identical to AidA from *R. solanacearum*. At present, there are no

homologues to AidA in the databases which can give information about a possible

function for this protein, however, the expression of the corresponding gene, *aidA,* has

been described to be regulated by quorum sensing in a [SolR-HSL]-dependent manner

in *R. solanacearum* (Flavier, *et al.,* 1997b). In addition, Northern analysis shows that

transcription of *aidA* is controlled by quorum sensing in *B. cepacia* ATCC 25416

(Figure 7), since in the quorum sensing-null mutant 25416-I there is no detectable

transcription of this gene. These results are supporting evidence that the above strategy

for the systematic investigation of genes controlled by quorum sensing in *B. cepacia*

was successful.

Nevertheless, it must be stressed that the present screening was not complete and a

larger one would be necessary to clone all the putative promoters of genes that are

regulated by quorum sensing. The incompleteness of this screening is supported since it

was not possible to identify clones harbouring the promoter of genes, other than *aidA,*

known to be controlled by quorum sensing as for example *cepI* or *pehA*. In addition, it

has been estimated that in *Pseudomonas aeruginosa* the total number of genes

controlled by quorum sensing could be 2-4 % of the 5000-6000 genes present in the

chromosome, that is around 200 genes (Whiteley *et al.,* 1999). The number of genes

controlled by quorum sensing can be even higher in *B. cepacia* since this organism has

a larger genome, with 7000-8000 genes.

A question that cannot still be answered with the strategy described in the present

chapter is whether there could be genes repressed by quorum sensing, since the

screening is based upon activation of transcription. The quorum sensing-dependent

repression of transcription has been described previously for some genes. Some examples are LuxR in *V. harveyi* (a HSL-dependent transcriptional activator that shares no homology with the *V. fischeri* LuxR), which makes an autorepression of transcription (Chatterjee *et al.*, 1996) and also HapR, which transcriptionally represses the expression of the Lys-type regulator AphA at high cell densities, by direct binding of HapR to the *aphA* promoter in *Vibrio cholerae* (Kovacikova & Skorupski, 2002).

As mentioned in Chapter 2, the CepR homologues among the *B. cepacia* complex share a 93-97 % amino acid identity (Table 2, chapter 2). This means that the same vector pSCR2 can probably be used in a similar way described in this chapter in order to obtain information about quorum sensing-controlled genes ([CepR-HSL]-dependant) from different genomovars in the *B. cepacia* complex and possibly also other bacterial species which produces HSL molecules which are recognized by CepR.

THE STATIONARY PHASE SIGMA FACTOR (RpoS) OF THE ONION

PATHOGEN *Burkholderia cepacia* GV. I TYPE STRAIN ATCC

25416.

4.1   INTRODUCTION

Bacteria frequently experience nutrient limitation in their natural environment, resulting in long periods of negligible growth or apparent dormancy. To overcome this condition, specialised metabolic states have evolved, as for example differentiation to spores in the Gram-positive *Bacillus subtilis* or the entry to what is normally referred as stationary phase in *E. coli* (Loewen & Hengge-Aronis, 1994). Consequently, the term stationary phase is used to denote a fixed physiological state regardless of what factors led to growth cessation. The stationary phase is synonymous with the starvation for only an ideal case in which the limiting nutrient leading to growth cessation can be specified; however even in laboratory culture conditions, the mechanism of cell growth cessation usually involves multiple factors (Ishihama, 2000).

The adaptive response to nutrient limitation during the entry into stationary phase involves a series of genetic switches that control the metabolic changes taking place. A common regulatory mechanism is the use of sigma factors, whose primary role is to bind the core RNA polymerase, conferring promoter specificity (Hengge-Aronis, 2002b; Ishihama, 2000; Loewen & Hengge-Aronis, 1994). RpoS (also known as σ^S and σ^{38}) is a master regulator of the stress response that directs transcription of a large number of genes involved in adaptation to nutrient limiting conditions, environmental stresses and production of virulence factors (Kojic *et al.*, 1999; Loewen & Hengge-Aronis, 1994; Suh *et al.*, 1999). In *Escherichia coli* and *Pseudomonas* spp. *rpoS* gene expression is induced as bacterial cultures enter stationary phase (Fujita *et al.*, 1994; Kojic & Venturi, 2001; Lange *et al.*, 1995).

In this chapter the identification and characterisation of the *rpoS* gene of *B. cepacia* ATCC 25416 is reported. This is the first report of *rpoS* in the *B. cepacia* complex, and only the second in the *β-Proteobacteria phylum*. The *B. cepacia rpoS* encodes for a 41 kDa protein that, as expected, did not display very high identity in its primary structure to RpoS belonging to *γ-Proteobacteria*. It is shown that, while important for the survival to heat shock and oxidative stress, it is not important in the survival to osmotic shock.

Another aspect covered in this chapter is the cross-regulation between RpoS and quorum sensing. Two lines of evidence suggest the existence of cross-regulation in *Pseudomonas aeruginosa*, but they are contrasting between each other. On the one hand, it has been shown that transcription of *rpoS* is activated by RhlR and C_4-HSL (Latifi, *et al.*, 1996), while for the other hand recent investigations suggest that quorum sensing may have no significant influence on *rpoS* transcription and indeed it is actually RpoS which influences transcription of *rhlI* (Whiteley *et al.*, 2000). In this chapter, it was observed that in *B. cepacia* ATCC 25416, RpoS was not involved in HSL production and that quorum sensing had a negative effect on *rpoS* transcription.

4.2   MATERIALS and METHODS

4.2.1   Bacterial strains, plasmids and media.

The plasmids used in this study are listed in Table 1. *B. cepacia* ATCC 25416 (or LMG 1222) (Burkholder, 1950) was routinely grown in Luria-Bertani broth or M9 minimal medium (Sambrook, *et al.*, 1989) at 30°C. *E. coli* strains used in this study included HB101 (Sambrook, *et al.*, 1989) and DH5α (Hanahan, 1983), grown in LB medium (Sambrook, *et al.*, 1989) at 37°C. Antibiotics were added as required at final concentrations of tetracycline 10 μg/ml (*E. coli*), 300 μg/ml (*B. cepacia*), gentamycin 10 μg/ml (*E. coli*), 300 μg/ml (*B. cepacia*), ampicillin 100 μg/ml (*E. coli*), kanamycin 50 μg/ml (*E. coli*) and 300 μg/ml (*B. cepacia*).

4.2.2   Recombinant DNA techniques.

Digestion with restriction enzymes, agarose gel electrophoresis, purification of DNA fragments, ligation with T4 DNA ligase, end filling with Klenow fragment of DNA polymerase, Southern hybridisation and transformation of *E. coli* were performed as described by Sambrook *et al.*, (1989). Analytical amounts of plasmids were isolated as described by Birnboim (1983), whereas preparative amounts were purified with Qiagen columns (Qiagen, Hilden, Germany). Total DNA from *B. cepacia* was isolated by sarcosyl-pronase lysis as described by Better *et al.* (1983). Tri-parental matings from *E. coli* to *B. cepacia* were performed with the helper strain *E. coli* (pRK2013) (Figurski & Helinski, 1979).

4.2.3   Purification, detection and visualisation of autoinducer (HSLs) molecules.

The purification, detection and visualisation of HSLs inducer molecules from culture supernatants were performed essentially as described by McClean *et al.* (1997) and Kojic & Venturi (2001). For quantification of CepR activity, overnight *E. coli* DH5α (pCRS1) cultures were normalised to an O.D. 600 nm of 0.1 in a volume of 20 ml LB containing the desired HSL at the desired concentration. Cultures were then grown with agitation at 37°C for 6 hrs and β-galactosidase activities were determined.

4.2.4   Cloning the *rpoS* gene of *B. cepacia* ATCC 25416.

The *rpoS* DNA sequences from cloned and characterised *Pseudomonas* spp. (Kojic, *et al.*, 1999) were compared with the genome sequence of *B. cepacia* Gv. III clinical isolate strain J2315 (www.sanger.ac.uk/Projects/B_cepacia/). The putative *rpoS* of *B. cepacia* J2315 was localised in chromosme 1.

Two synthetic primers were designed from the conserved regions (rpoS-P3 5-CTGCTCGACCTGATCGA-3, and rpos-P6 5-AGGCTGCTCGCGGGATC-3) and used in a PCR-amplification reaction. PCR amplification was performed in 50 μl volume, containing primers (0.5 μM each) deoxynucleotide triphosphates (200 μM each), chromosomal DNA (100 ng), and Vent DNA polymerase (0.5 U) in the buffer recommended by the manufacturer (New England Biolabs). After an initial incubation at 95°C for 5 min, 30 rounds of temperature cycling were performed under the following conditions: 95°C for 60 s, 55°C for 60 s, and 72°C for 60 s. The amplified fragment was cloned as a 300 bp fragment in pUC18 yielding pRPC-1. This fragment

was then used as probe for the identification of *rpoS*. About 1000 colonies of *E. coli*, harbouring the cosmid library of *B. cepacia* ATCC 25416, were used in a colony blot experiment. A cosmid, designated pCOSRPOS-2C, was identified. Subsequent analysis of this cosmid by Southern hybridisation was made, using several restriction enzymes for the digestion of pCOSRPOS-2C DNA and using the 300 bp PCR product mentioned above as probe. In this way, the *rpoS* gene was localised within a 5.5 kbp *Sma*I fragment (Figure 4) and in part in a 1 kbp *Pst*I fragment, these fragments were cloned in the corresponding sites in pBluescript KS yielding pRBS-2 and pRBS-3 respectively.

The *rpoS* promoter transcriptional fusion was constructed as follows. A 2 kbp *Bam*HI fragment from pRBS-2 was cloned into the *Bgl*II site of the promoter probe vector pMP190 (Spaink, *et al.*, 1987), yielding pRPR2. The correct orientation of the fragment was checked by restriction enzyme analysis.

4.2.5   Reporter gene fusion assays

β-galactosidase activity was determined as described by Miller (1972) with the modifications of Stachel *et al.* (1985).

4.2.6   Construction of a *B. cepacia* ATCC 25416 *rpoS* knock out mutant.

The kanamycin resistant gene from pUC4K was cloned as a *Bam*HI fragment in the corresponding site of pRBS-3, creating pRBS-3Km, resulting in an interruption of the cloned *rpoS* gene of *B. cepacia* ATCC 25416. The pRBS-3Km was used to transform *E. coli* DH5α (pCOSRPOS-2C) and the resulting *E. coli* DH5α (pCOSRPOS-2C) (pRBS-

3Km) was grown overnight. The culture was then used in a tri-parental conjugation into *P. putida* WCS358 (Geels & Schippers, 1983) using *E. coli* (pRK2013) as helper. After appropriate selection, pCOSRPOS-2CKm was selected. Transfer of Km cassette by double cross-over homologous recombination from pRBS-3Km to pCOSRPOS-2 was verified by Southern analysis. The plasmid pCOSRPOS-2CKm was then used in a marker exchange technique, as described above, in order to introduce insertion mutations site-specifically with the *rpoS* gene of *B. cepacia* ATCC 25416. The fidelity of the marker exchange event in the *B. cepacia rpoS::Km* mutant was confirmed by Southern analysis (Figure 4). This mutant was designated *B. cepacia* 25416-RPOS.

4.2.7 Stress response assays.

B. cepacia was grown overnight in LB at 30 °C, washed in M9 medium and diluted in M9 to a density of 7000 cfu/ml. To determine the ability to survive heat shock, one milliliter of the diluted culture was placed in a pre-warmed tube at 50 °C, and the viability was determined by taking periodic aliquots and plating them directly on LB plates. To measure the sensitivity to osmotic shock, diluted cells were resuspended in M9 medium with 2 M NaCl. These cells were incubated at 30 °C, periodic aliquots were taken and plated on LB plates to determine cfu.

The sensitivity to hydrogen peroxide was measured on cells grown for 16 h in LB at 30 °C, plated on LB plates. Sterile Whatman filter discs impregnated with 10 μl of 30% H_2O_2 were placed on top of bacterium-seeded plates and incubated at 30 °C for 16 h. The zones of inhibition were measured in millimeters.

4.2.8 Protein expression, purification and antibodies against RpoS.

The *rpoS* from *B. cepacia* ATCC 25146 was amplified by PCR using two

oligonucleotides (PQERPOSATG, 5'-GGTACCATGCCGAAATCGAAGCGCCAC

GAG-3'; PQERPOSEND, 5'-AAGCTTACAGAACGGCGTCCTTGCGCACGC-3').

PCR amplification was performed in 50 µl volume, containing primers (0.5 µM each)

deoxynucleotide triphosphates (200 µM each), pRBS-2 DNA (10 ng), and Vent DNA

polymerase (0.5 U) in the buffer recommended by the manufacturer (New England

Biolabs). After an initial incubation at 95°C for 5 min, 35 rounds of temperature cycling

were performed under the following conditions: 95°C for 30 s, 65°C for 60 s, and 72°C

for 90 s. The amplified fragment was cloned as a *Kpn*I-*Hind*III fragment into the

corresponding sites of pQE30, thus yielding pQERPOSBC. The 6xHis-RpoS was

expressed in *E. coli* M15 (pREP-4) and purified according to the instructions provided

by the supplier (Qiagen, Hilden, Germany). Polyclonal antibodies against RpoS were

produced in rabbits by injecting the purified 6xHis-RpoS.

Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane

(Immobilon-P; Milli-pore Corp.) using a tank system according to the manufacturer's

instructions. The membrane was subjected to Western blot analysis using rabbit anti-

RpoS serum (1:500) in PBS. After incubation with the secondary horseradish

peroxidase-labeled antibody, the proteins were detected either with 3-3'-

diaminobenzidine tetrahydrochloride tablets (Sigma, St. Louis, Mo.) or with ECL

western blotting detection system (Amersham Pharmacia Biotech).

4.2.9   DNA sequence determination, analysis and database accession number.

DNA sequence of the *rpoS* gene was determined using plasmids pRBS-2 and pRBS-3

as templates. Nucleotide sequences were determined by the dideoxy chain-termination

method (Sanger, *et al.*, 1977) using [^{35}S] dATPαS for labelling and 7-deaza-dGTP

(Pharmacia) instead of dGTP. Analysis of the sequence was performed with EditSeq

and MapDraw software (DNASTAR, Inc.). BLASTX and BLASTN programs were

used to search the non-redundant sequence database for homologous sequences

(Madden, *et al.*, 1996).

The sequence of the *B. cepacia* ATCC 25416 *rpoS* gene has been deposited in the

GenBank/EMBL/DDBJ database under accession numbers AJ457984.

4.2.10 Primer extension analysis.

Total RNA was extracted from 2.5 ml of mid log *B. cepacia* growing cells (OD $_{600nm}$

3.0), using the RNeasy Mini Kit according to the instructions provided by the supplier

(Qiagen, Hilden, Germany). RNA concentration was estimated spectro-photometrically.

For primer extension, about 25 μg of total RNA were annealed in hybridization buffer

(150 mM KCl, 10 mM Tris-Cl pH 8.0, 1 mM EDTA) with a [γ-^{32}P]-end-labelled

oligonucleotide (BCPRPOS: 5'-GCTGCCGTGTCGCT CGATGC-3') for 90 min at 65

°C. Extension was performed by adding the reaction mix (30 mM Tris-Cl pH 8, 15 mM

MgCl2, 8 mM DTT, 250 μM dNTPs and 2 U AMV reverse transcriptase (Promega))

and incubating for 1h at 42 °C. After this incubation period, samples were treated with

RNAse (2 μg RNAse A), extracted with phenol/chloroform/isoamyl alcohol (25:25:1),

and further precipitated with ethanol. Pellets were then resuspended in 5 μl stop/loading

dye and fractionated on a 8 % polyacrylamide denaturing gel containing 7M urea. A

DNA sequence ladder was obtained with the M13 specific primer and pBluescript KS as

template, using $[\alpha\text{-}^{35}S]$ dATP for labelling and 7-deaza-dGTP (Pharmacia) instead of

dGTP, following the instructions provided by the supplier.

4.2.11 Plasmids used.

Table1. Plasmids used in this study.

Plasmid	Description[a]	Source or refrence
pBluescript KS	Ap^r; ColE1 replicon	Stratagene
pUC18	Ap^r; ColE1 replicon	(Yanisch-Perron, et al., 1985)
pMP190	Cm^r; IncQ; promoter probe vector	(Spaink, et al., 1987)
pUC4K	Ap^r; pBR322 replicon	Amersham Pharmacia
pRK2013	Km^r;Tra$^+$; Mob$^+$; ColE1 replicon	(Figurski & Helinski, 1979)
pPH1Jl	Gm^r; IncP1	(Beringer, et al., 1978)
pLAFR3	Tc^r; Broad-host-range cloning vector; IncP1	(Staskawicz, et al., 1987)
pQE30	Ap^r; ColE1 replicon; 6xHis expression vector	Qiagen
pQF50	Ap^r; Broad-host-range vector; pRO1600 replicon	(Farinha & Kropinski, 1990)
pRPC-1	Ap^r; pUC18 containing a 300 bp PCR fragment of *rpoS* gene	This study
pCOSRPOS-2C	Tc^r; pLAFR3 containing *B. cepacia* DNA	This study
pRBS-2	Ap^r; pBluescript KS containing 5 Kbp *Sma*I from pCOSRPOS-2C	This study
pRBS-3	Ap^r; pBluescript KS containing 1 Kbp *Pst*I from pCOSRPOS-2C	This study
pRBS-3Km	Ap^r; Km^r; pRBS-3 with a Km cassette in *rpoS* gene	This study
pCOSRPOS-2CKm	Tc^r; Ap^r; Km^r; pCOSRPOS-2C with a Km cassette in *rpoS* gene	This study
pQERPOSBC	Ap^r; *rpoS* cloned in pQE30	This study
pRPR2	Cm^r; P*rpoS-lacZ* fusion in pMP190 promoter probe vector	This study

[a] Ap^r, Cm^r, Gm^r, Km^r, Tc^r: resistant to ampicillin, chloramphenicol, gentamycin, kanamycin and tetracycline, respectively.

4.3   RESULTS

4.3.1   Identification of the stationary phase *rpoS* gene of *B. cepacia* ATCC 25416.

In order to identify the *rpoS* gene of *B. cepacia* ATCC 25416, the genome of *B. cepacia* J2315 was compared to previously cloned DNA sequences of *rpoS* from *Pseudomonas spp.*. Synthetic primers were designed from the conserved regions and used in a PCR reaction with genomic DNA of *B. cepacia* ATCC 25416 as template (see Materials and Methods for details). Consequently, a cosmid that contains the *B. cepacia* ATCC 25146 *rpoS* gene was identified. The nucleotide sequence was determined; *rpoS* consists of 1089 nucleotides, encoding a protein of 362 amino acids with an estimated molecular mass of 41kDa. As can be seen in Table 2, it displayed the highest amino acid identity (74 %) to the RpoS of *Ralstonia solanacearum*, whereas approximately 50 % identity with the RpoS sigma factors of γ-*Proteobacteria* (eg. *P. aeruginosa*, *E. coli* and *Vibrio cholerae*). A genetic relatedness tree with RpoS amino acid sequences is depicted in Figure 1.

Table 2. RpoS amino acid identity in different bacterial species.

Organism	Identity[a]	MW (KDa)
Pseudomonas aeruginosa PAO1	54	38.2
Vibrio cholerae	46	38.5
Salmonella typhimurium	47	37.9
Escherichia coli K12	47	37.9
Yersinia enterocolitica	50	37.9
Shigella flexneri	50	41.4
Ralstonia solanacearum	74	41.9
Pseudomonas putida WCS358	48	38.2

[a]Identity expressed as percentage, in relation to RpoS from *B. cepacia* ATCC 25416.
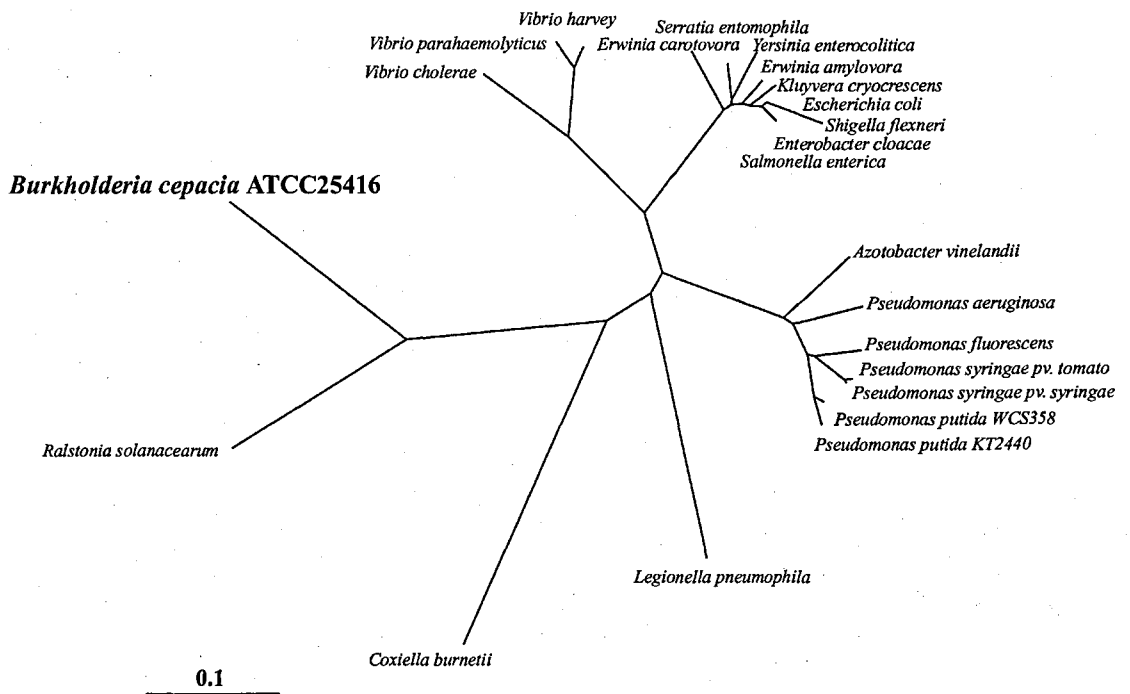
Figure 1. Genetic relatedness of experimentally identified RpoS homologues in *Proteobacteria*. Amino acid sequences were aligned using the ClustalW (http://www2.ebi.ac.uk/clustalw) program. The phylogenetic tree was assembled using the Treeview program (http://taxonomy.zoology.gla.ac.uk/rod/treeview). The RpoS homologues were from *Escherichia coli* (EMBL P13445), *Pseudomonas aeruginosa* (P45684), *P. syringae* (Q9RBQ6), *Ralstonia solanacearum* (O52607), *P putida* WCS358 (Q9EX90), *P. putida* KT2440 (P77927), *Vibrio cholerae* (Q9JQ13), *P. syringae pv. tomato* (O69079), *Legionella pneumophila* (Q9S4T1), *V. parahaemolyticus* (Q9X6S5), *Coxiella burnetii* (Q9KI19), *V. harvey* (Q9ANS6), *Azotobacter vinelandii* (Q93AG3), *Enterobacter cloacae* (O08372), *Erwinia carotovora* (Q9REC2), *E. amylovora* (O69431), *Kluyvera cryocrescens* (O32708), *Serratia entomophila* (Q59904), *Salmonella enterica* (P37400), *Shigella flexneri* (P35540), *Yersinia enterocolitica* (P47765), *P. fluorescens* (Q59664), *Burkholderia cepacia* ATCC 25416 (AJ457984).

4.3.2   Identification of the *rpoS* transcriptional start site

The *rpoS* promoter was further characterised. Total RNA was isolated from *B. cepacia*

and used in primer extension experiments to determine the transcriptional start site of

rpoS. As depicted in Figure 2a, a single band was obtained for the *rpoS* mRNA

extension product. Using the sequence of pBluescript KS as DNA ladder, the start of

transcription was deduced to be located at 463 bp upstream from the *rpoS* translational

start codon, within the *nlpD* gene (Figure 3a). It was also possible to identify the

putative -10, -35 and SD elements in the sequence of the promoter, as depicted in Figure

2b.

4.3.3   Insertional inactivation of *rpoS* in *B. cepacia* ATCC 25416.

In order to investigate the role that *rpoS* played in *B. cepacia*, this gene was

insertionally inactivated in strain ATCC 25416. Plasmid pCOSRPOS-2CKm, which

contained a Km resistance gene cloned in the *rpoS* gene, was used in a marker exchange

experiment to introduce an insertion mutation site-specifically within the *rpoS* gene of

strain ATCC 25416. This experiment resulted in the construction of an *rpoS*::Km

knock-out mutant called *B. cepacia* 25416-RPOS. A genetic map of the region coding

for *rpoS*, and the interrupted locus is shown in Figure 3a. The fidelity of the marker

exchange was confirmed by Southern analysis, as shown in Figure 3b.

(a)
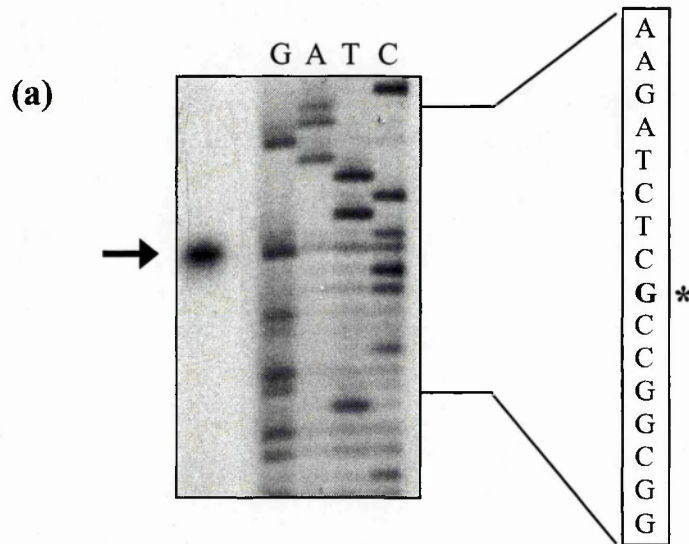
(b)

```
GACCAGCTGCTGCGCGTGTCGCCGCAGGCCGGTGCCGCTGTCGCGGGCACCCAGGTCGCCGCGCCGATCGGGGGCGCCGC
                                                                       -35
                          +1
CGTCGCTACCGCGCCGCTCAGTAGCGGCCCGGCAACGCCG...411bp...TGTCGATCCGCTGAAGTATTTGCCGCCTC
                         -10

AATAACCGAGACGACCATGCCGAAATCGAAGCGCCACGAGCCGCAAGCCGAGTCTGAGAAGATCAGTCGTGCCACGCAAG
         S.D.
```

Figure 2. The *rpoS* promoter analysis. (a) The arrow indicates the primer extension product, visible as a single band. At the right side, pBluescript KS sequence is shown; evidenced in bold and with an asterisk is the nucleotide from which the initiation of transcription was deduced. (b) putative -10 and -35 sites, the ATG translation start codon, and the Shine-Dalgarno sequence (S.D.) are depicted. The arrow indicates the deduced start of transcription for *rpoS*.
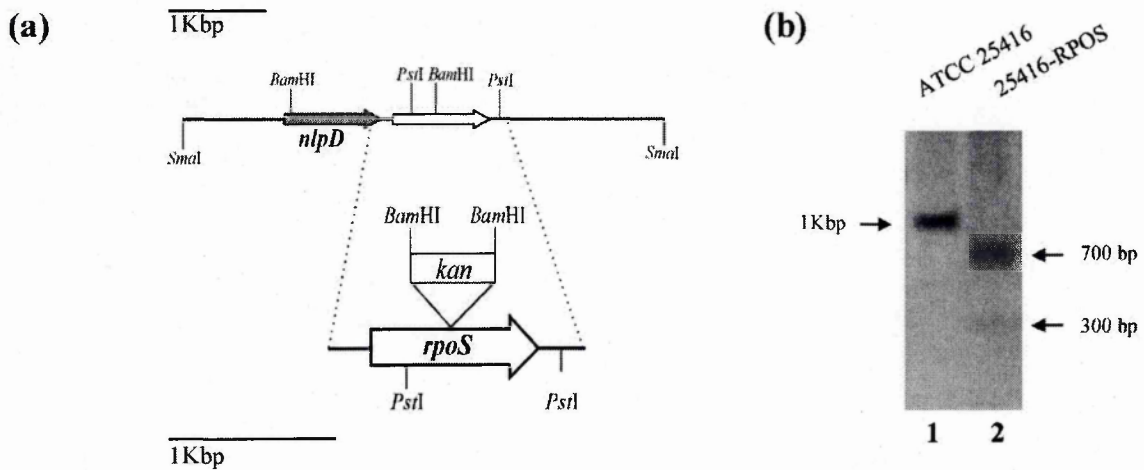
Figure 3. Construction of *rpoS* genomic mutant (a) Map of the 5.5 Kbp *Sma*I DNA fragment from *B. cepacia* ATCC 25416 isolated in this study. Shown are several enzyme restriction sites and the location of the *rpoS* gene within this fragment. Shown is also the position where the kanamycin resistance gene (*kan*), as a *Bam*HI fragment derived from pUC4K, was cloned in the corresponding site of the *rpoS* gene to create pLCIKm. (b) Construction of *B. cepacia* 25416-RPOS, as verified by Southern analysis. The *rpoS* gene was replaced in the chromosome with the cloned *rpoS::Kan*. Lane 1 is genomic DNA from 25416 and lane 2 is genomic DNA from 25416-RPOS, both digested with *Pst*I. These were then probed with a 1 Kbp *Pst*I fragment containing the sequence of *rpoS*. In the parent strain (lane 1) a fragment of approximately 1 kbp was observed whereas in the mutant this fragment was divided in two bands of 700 and 300 bp respectively.

4.3.4   Characterisation of the *rpoS* knock-out mutant from *B. cepacia* ATCC 25416

The RpoS sigma factor is known to confer cross-protection against several stresses in Gram-negative bacteria (Gerard, *et al.*, 1999; Ramos-Gonzalez & Molin, 1998; Suh, *et al.*, 1999). Therefore, the response of *B. cepacia* 25416-RPOS to various environmental conditions was tested. The resistance of the *rpoS* mutant against heat shock, hydrogen peroxide and increased osmolarity was tested and compared to the wild type parent strain . When stationary phase cultures were exposed to a sudden shift in temperature from 30 °C to 50 °C, the *rpoS* mutant was more sensitive to this heat shock (Figure 4a). Similarly, RpoS mediated protection against hydrogen peroxide and in stationary phase cells of *rpoS* mutants were significantly more sensitive when compared to wild type bacteria (Figure 4b). When the cells were exposed to an increase in osmotic pressure caused by the addition of high concentration of salt, the *rpoS* mutant was only slightly more sensitive after a prolonged period of incubation (Figure 4c).

4.3.5   RpoS concentration increases in a growth phase-dependent manner.

RpoS cellular concentration was followed over the growth phase, using polyclonal antibodies against a purified RpoS of *B. cepacia* ATCC 25416 (see Materials and Methods). Protein extracts were made at various time points and 15 μg of total protein were used in Western immunoblot analysis (see Material and Methods). As can be seen on Figure 5, RpoS cellular concentration continued to increase until entry in stationary phase.

(a)

(b)
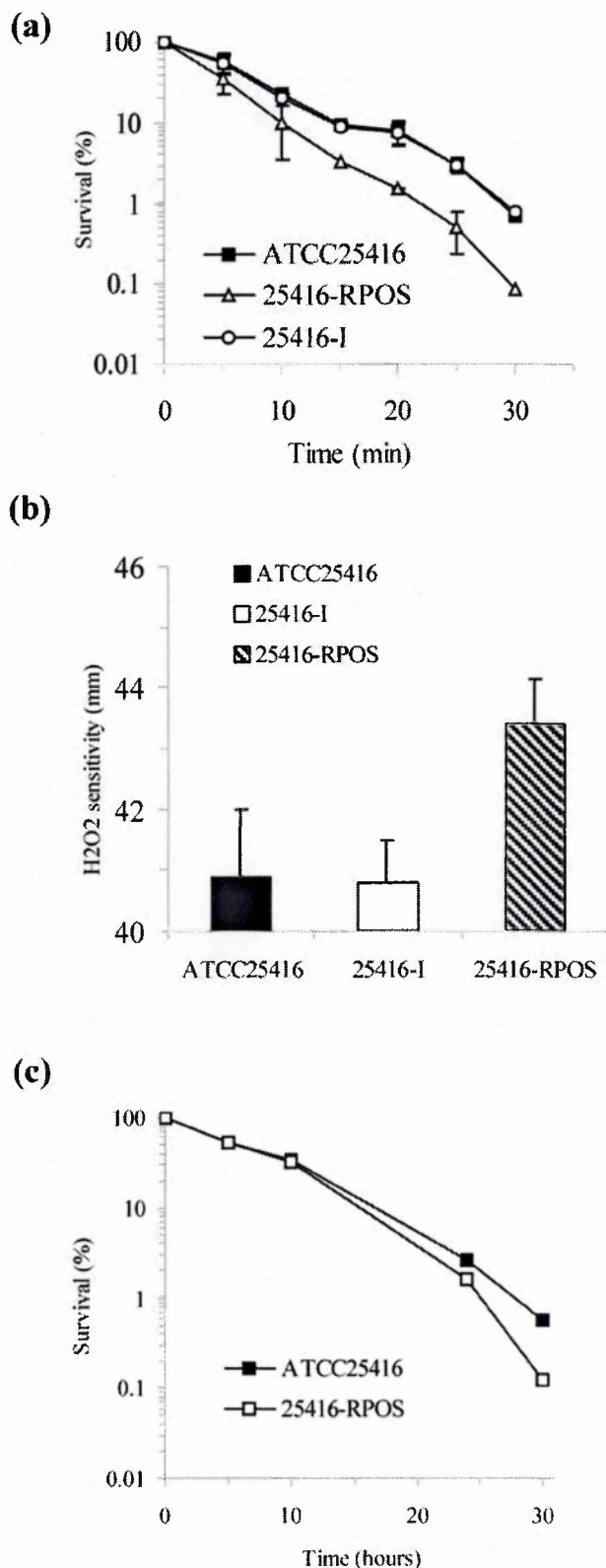
Figure 4. **Effect of RpoS on stress responses. (a)** Response to heat shock (50^{0}C). Survival is expressed as a percentage of the number of cfu at time 0, measured for WT, 25416-RPOS and 25416-I. **(b)** Effect of *rpoS* mutation on oxidative stress. The sensitivity to H_2O_2 was measured on cells grown for 16 hours in LB at 30^{0}C. The zones of inhibition were measured in millimetres. Filled bars: WT strain; open symbols: *B. cepacia* 25416-RPOS derivative. **(c)** Response to osmotic shock (2M NaCl). Survival is expressed as a percentage of the number of cfu at time 0 for WT and 25416-RPOS derivative..

(c)
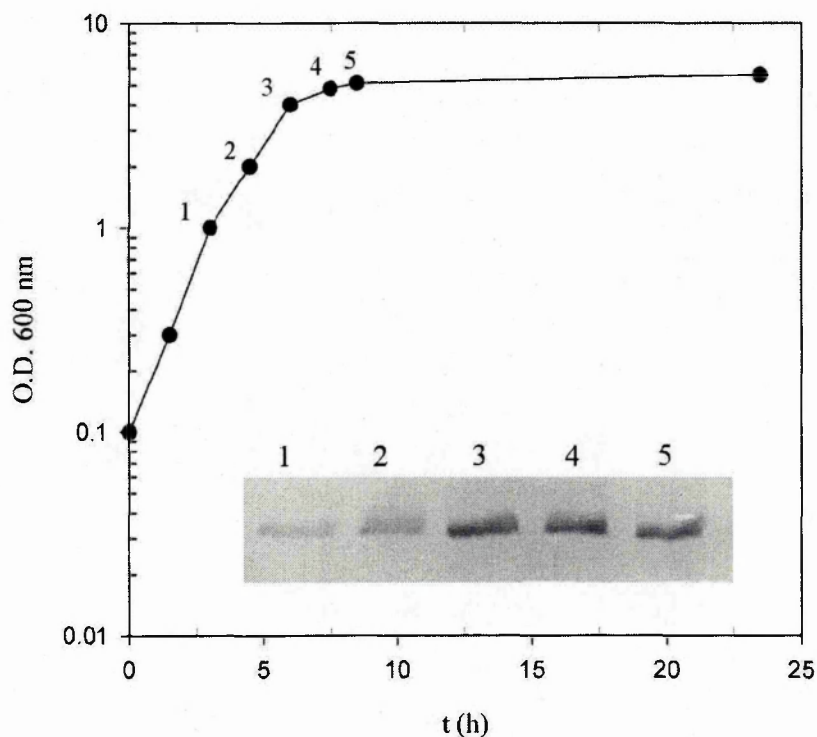
Figure 5. Growth phase-dependent increase in cellular RpoS concentration.
B. cepacia ATCC 25146 was grown on LB medium. Samples were taken along
the curve as indicated by the numbers, normalized for equal total protein
concentration (15 µg) and subjected to Western immunoblot analysis with rabbit
anti-RpoS serum (inside box).

4.3.6   Do RpoS and quorum sensing cross-regulate each other in *B. cepacia*?

It was of interest to investigate whether RpoS and quorum sensing cross-regulate each
other since the genes regulated by these two systems are maximally expressed at
stationary phase.

In order to determine whether *rpoS* influenced the accumulation of HSL signal
molecules, the HSL production in 25416-RPOS was quantified and compared to the

values obtained using the wild type strain. Signal molecules were extracted from spent supernatants at different growth stages and the extracts assayed with the sensor *E. coli* (pSCR1) (see also Chapter 2).

Figure 6a depicts the results showing that the *B. cepacia* mutant lacking RpoS synthesises approximately the same amount of signal molecules produced by the wild type parent strain at different growth stages, demonstrating that the absence of RpoS does not influence the production of HSL molecules.

In order to determine whether the quorum sensing system is regulating *rpoS* expression, the *rpoS* promoter was cloned as a 2 kb *Bam*HI fragment from pCOSRPOS-2C in the *Bgl*II site of β-galactosidase promoter probe vector pMP190 (Spaink, *et al.*, 1987), obtaining pRPR2. *rpoS* promoter activity was then assayed at different growth stages in the *cepI* mutant 25416-I and compared to promoter activity obtained in the wild type strain. As depicted in Figure 6b, *rpoS* expression in wild type strain ATCC 25416 was relatively constant during early, exponential and stationary phase. It was then determined that in the *cepI* mutant 25416-I, *rpoS* expression increased 2-3 fold in all growth stages, and that this increase in activity was retained when 100 or 1000 nm of C_8-HSL was added to the growth media.

Protein extracts of *B. cepacia* ATCC 25416; *B. cepacia* 25416-I and *B. cepacia* 25416-I (pMPIR) where made from overnight cultures. Similar amount of protein (15 μg) was subjected to Western immunoblot analysis. As depicted in Figure 6c, RpoS levels in stationary phase culture were indistinguishable from the wild type in the *B. cepacia* derivatives.
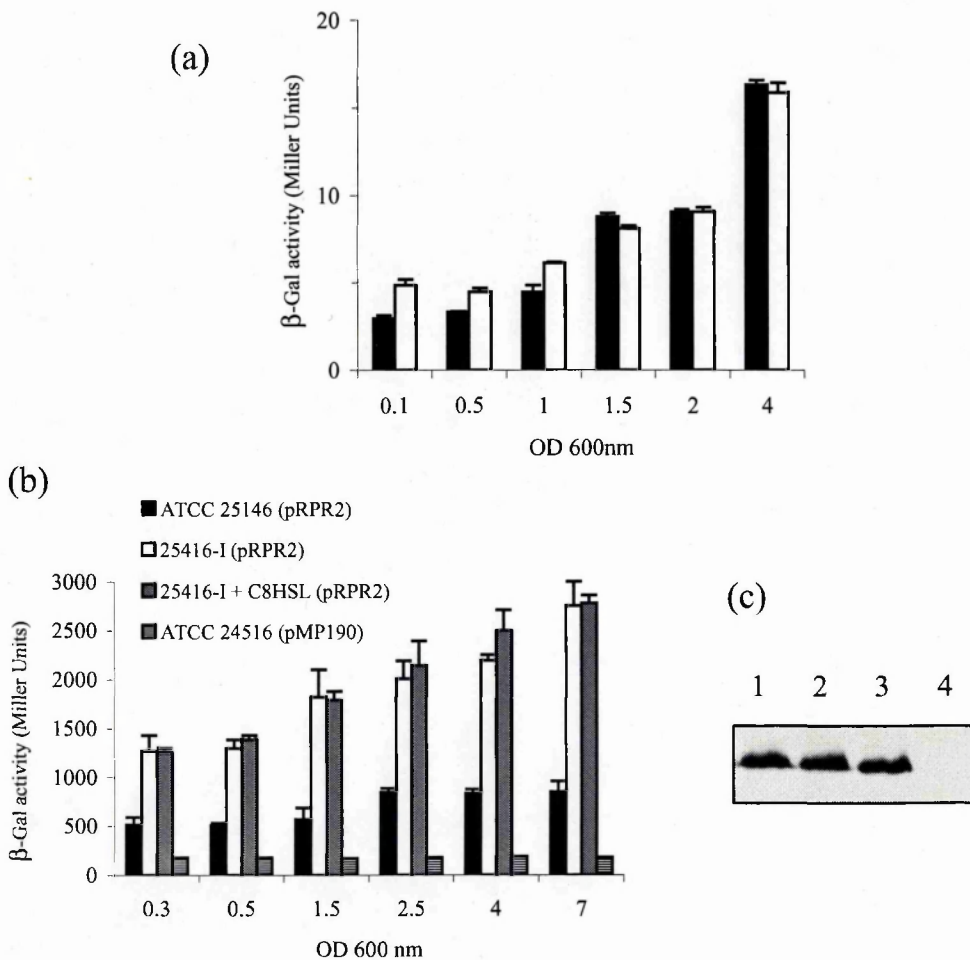
Figure 6. RpoS and quorum sensing cross-regulation (a) Production of HSLs at different growth stages from *B. cepacia* ATCC 25416 (filled bars) and from *B. cepacia* 25416-RPOS derivative (open bars). Values were determined as described in Materials and Methods. **(b)** *rpoS* promoter activity at different growth stages measured from *B. cepacia* ATCC 25416 (pRPR2) (filled bars), from *B. cepacia* 25416-I (pRPR2) (open bars), from *B. cepacia* 25416-I (pRPR2) + 100 nM C$_8$-HSL (shaded bars) and *B. cepacia* ATCC 25416 (pMP190) (striped bars). Values were determined as described in Materials and Methods. **(c)** Western immunoblot analysis, using rabbit anti-RpoS serum . Protein extracts were made from overnight cultures. Lane 1, *B. cepacia* ATCC 25416; lane 2, *B. cepacia* 25416-I; line 3, *B. cepacia* 25416-I (pMPIR); line 6, negative control using *B. cepacia* 25416-RPOS derivative.

4.4 DISCUSSION

4.4.1 Identification and characterization of *rpoS* in *B. cepacia*.

The stationary phase sigma factor RpoS (also known as σ^s and σ^{38}) has been implicated in the change in gene expression necessary for the adaptation to stationary phase in several Gram-negative bacteria (Gerard, *et al.*, 1999; Ramos-Gonzalez & Molin, 1998; Suh, *et al.*, 1999), and it has also been shown to be involved in the regulation of virulence factors (Iriarte et al., 1995; Sarniguet et al., 1995; Corbell & Loper, 1995). In this chapter, RpoS from *B. cepacia* was identified and partially characterized. The *rpoS* gene is 1089 bp in length, coding for a protein of 41 kDa. This molecular weight is slightly higher to what was originally described in *E. coli* and other bacteria belonging to the γ-subdivision. This difference can probably be explained since *B. cepacia* belongs to the β-*Proteobacteria phylum* and consequently it displayed the highest amino acid identity (78 %) with RpoS of *R. solanacearum*, up to now the only other RpoS to be experimentally identified in a β-*Proteobacteria* organism (Figure 1).

Primer extension studies revealed that the *B. cepacia rpoS* promoter is located within the *nlpD* gene (which encodes for a lipoprotein of unknown function) (Figure 3a). The *rpoS* transcript contains an untranslated 5' region of 463 bp (Figure 2b). These two features, an unusually long untranslated 5' region and the promoter within the *nlpD* gene, have been previously observed in other Gram-negative bacteria such as *E. coli* (Lange, *et al.*, 1995; Takayanagi *et al.*, 1994) and *Pseudomonas* (Kojic *et al.*, 2002). It has been suggested in *E. coli* that this 5' region in the mRNA serves for regulation of

the expression of *rpoS* at the post-transcriptional level, since it is capable of forming a stem-loop structure that blocks the ribosome-binding site and prevents translation. Several point mutations that weaken the pairings within the double-stranded stem structure lead to the constitutive expression of *rpoS* in *E. coli* (Brown & Elliott, 1997). DsrA and RprA are small RNAs that can overcome the hairpin stem-loop, thus positively regulating *rpoS* expression (Majdalani *et al.*, 2001). It cannot therefore be excluded that the long 5' leader region, present in the *B. cepacia rpoS* mRNA, can be used for regulation in an analogous way (see below).

In addition, it was observed that the potential −35 and −10 promoter elements of the *rpoS* gene are separated by 24 nucleotides, a longer distance compared with the homologous genes present in *E. coli* or *P. aeruginosa*, with 17 and 20 nucleotides, respectively (Fujita, *et al.*, 1994; Hengge-Aronis, 2002b). This distance is unusual, and point mutations in this region could reveal if this elements are functional or if there was an error assigning the exact position of the −35 and −10 promoter elements.

4.4.2   Role of RpoS in stress survival in *B. cepacia*.

B. cepacia is an extremely versatile organism, and its ability to resist, adapt, and survive in a wide variety of environments likely contributes to its opportunistic pathogenic behaviour. In order to study the stress responses of this organism, an RpoS deficient mutant was constructed and characterised.

Several studies have associated RpoS with the resistance of bacteria to environmental stresses such as exposure to H_2O_2, shift in temperature and salinity (Hengge-Aronis,

2002b). The mechanisms of stress adaptation can be diverse in different organisms. For example, while in *P. aeruginosa* PAO1 and *P. putida* WSC358 RpoS is important to survival in all stress conditions mentioned above (Kojic, *et al.*, 1999; Suh, *et al.*, 1999), in *P. putida* mt-2 RpoS is regulating the oxidative stress and salinity shift but not the heat shock response (Miura *et al.*, 1998).

In this chapter, it is shown that in *B. cepacia* RpoS is necessary for adaptation to heat and oxidative stress, whereas it was not involved in osmotic shock adaptation (Figure 4). Protection against H_2O_2 is mediated by catalases (KatA and KatB) and the expression of the genes encoding these enzymes is modulated by RpoS (Loewen & Hengge-Aronis, 1994; Miura, *et al.*, 1998). In addition to RpoS it has been shown in *P. aeruginosa* that quorum sensing is also involved in the expression of *katA* (Hassett *et al.*, 1999), since a *P. aeruginosa* strain deficient in the quorum sensing system is more sensitive to oxidative stress. The results presented in this chapter show that quorum sensing in *B. cepacia* is not controlling the expression of catalase(s), since the quorum sensing mutant derivative 25416-I is resistant to H_2O_2 when compared to the RpoS deficient strain 25416-RPOS (Figure 4b). This strongly suggests that in *B. cepacia* the influence of quorum sensing in survival to oxidative stress, consequently in catalase expression, is minimum or not detectable, and that RpoS is the major regulator of the expression of the genes encoding these enzyme(s). Similarly, derivative 25416-I is also resistant to heat shock at levels of the wild type strain, meaning that quorum sensing does not influence the survival to shift in temperature (Figure 4a).

4.4.3   Cross-regulation of quorum sensing and RpoS in *B. cepacia*.

It was of interest to study if RpoS and quorum sensing cross-regulate each other since

the gene(s) regulated by these two systems are maximally expressed at stationary phase.

It was determined that a genetic background without RpoS has no influence on the

accumulation of the HSL molecules produced by *B. cepacia* (Figure 6a), on the other

hand *rpoS* promoter activity is positively influenced by the absence of the quorum

sensing system resulting in a two-fold increase in *rpoS* promoter activity (Figure 6b).

This doubling in promoter activity did not reflect into an increased resistance towards

RpoS regulated stresses (see above), this might be because increasing the promoter

activity does not automatically result in increasing amount of RpoS protein as post-

transcriptional regulation of RpoS might occur (Hengge-Aronis, 2002b). This increase

in *rpoS* promoter activity in the *cepI* mutant 25416-I could not be reduced to wild type

levels by the addition to the growth media of synthetic C_8-HSL (Figure 6b). The reason

for this is not known, in addition, it was observed that *rpoS* promoter activity was

higher in *B. cepacia* 25416-I derivative already at low cell densities and activity

increased as the cells were entering stationary phase. In the wild type strain however,

the expression of *rpoS* remained relatively constant during bacterial growth, this is in

contrast to *rpoS* regulation in *Pseudomonas* where transcription dramatically increases

at the onset of stationary phase (Kojic & Venturi, 2001; Loewen & Hengge-Aronis,

1994; Venturi, 2003). However, it is similar to what happens in *E. coli*, since RpoS

levels are mainly controlled at the post-transcriptional and post-translational level

(Hengge-Aronis, 2002b). The observation that *rpoS* transcription in *B. cepacia* is

relatively constant throughout growth phase (Figure 6b), whereas Western analysis using anti-RpoS antibodies demonstrated that cellular RpoS accumulates upon entry in stationary phase (Figure 5), points towards post-transcriptional regulation of *rpoS* in *B. cepacia*.

In *R. solanacearum* it has been reported that RpoS regulates the HSL production by the decrease in *solR* and *solI* expression (Flavier, *et al.*, 1998), similarly in *P. aeruginosa* quorum sensing and *rpoS* cross-regulate each other (Latifi, *et al.*, 1996; Whiteley, *et al.*, 2000). It would be interesting to determine how quorum sensing regulates *rpoS* and if the two systems are involved in the regulation of similar phenotypes in *B. cepacia*.

SUMMARISING DISCUSSION

5.1 SUMMARISING DISCUSSION

It has become clear in the last decade that bacteria are able to communicate and to regulate gene expression based on sensing the cell population density. This phenomenon, broadly accepted among the scientific community, is called quorum sensing and it consists in the ability of synthesis, release, detection and response to threshold concentration of diffusible molecules, synthesised by the bacterial cells. Quorum sensing systems have been detected in several organisms and the genetic elements coding for such systems, have been isolated and characterised for the majority of them. The quorum sensing systems in Gram-negative bacteria are in most of the cases composed on the one hand by proteins members of the LuxI/R family, and on the other hand by the diffusible signals that are often N-acyl-homoserine lactones. The system works in such a way that when the diffusible signal, synthesised by the LuxI family protein, accumulates in the growth media above a critical concentration, the transcriptional regulator belonging to the LuxR family interacts with these molecules, thus activating transcription. Because the concentration of signal molecules reach the induction threshold only when the culture exceeds a critical cell density, these systems are thought to have evolved to allow bacteria to detect cell density and thus optimise the expression of functions that are unproductive when undertaken by an individual bacterium, but become effective when made by the group. The ability to coordinate gene expression in a cell-density-dependent fashion has several advantages as for example the regulation of virulence determinants in pathogenic microorganisms, that makes possible to evade host defences until a high cell density is reached; only then the

virulence determinants are expressed, allowing bacteria to make a concerted attack to overwhelm the host defences. In this sense, it has also become evident that an intact quorum sensing system is necessary not only for the maximal expression of some virulence factors, but also to cause disease (Pearson *et al.*, 1997; Wu *et al.*, 2001).

The aims of the investigations conducted through the present thesis were to study the quorum sensing system of the gram-negative bacteria *Burkholderia cepacia* genomovar I type strain, ATCC 25416.

It was possible to detect two major HSL from the supernatants of this strain, corresponding to C_6-HSL and C_8-HSL, and a third signal probably corresponding to C_7-HSL. The genetic determinants coding for the quorum system, *cepI/R*, were isolated and characterised, and it was observed that when *cepR* was interrupted in the chromosome, there is a significant reduction in the levels of HSL production. This phenomenon is explained by a positive autoregulation of the system since CepR would increase the transcription levels of *cepI* in a quorum sensing fashion, which is in accordance to what has been in the clinical isolate, belonging to genomovar III, *B. cepacia* K56-R2 (Lewenza *et al.*, 1999). Interestingly, when *cepI* was interrupted it was not possible to detect any HSL in the culture supernatants of the strain in study, thus suggesting that CepI is needed for the synthesis of all the HSL molecules detected. On other hand, the *B. cepacia* ATCC 25146 quorum sensing genes were used to construct a sensor plasmid in an *E. coli* background, that detects specifically the signals produced by this strain. It was determined that CepR uses preferentially C_8-HSL, rather than C_6 or C_7-HSL, for activating transcription of the reporter gene. Moreover, the CepR activity was higher with long chain HSL (C_{10} and C_{12}), than with short chain HSL (C_4, C_6, C_7), when

compared to the activity obtained with C_8-HSL. It would be interesting to elucidate a possible specific role in quorum sensing for C_6 and C_7-HSL, which can be found in the supernatant of *B. cepacia,* but only weakly activate the CepR-based biosensor pSCR1. Recently, *in silico* analysis conducted over the genome of two completely sequenced rhizobial strains, have revealed in *Sinorhizobium meliloti* the presence of one gene similar to the LuxI family and several showing some similarity to LuxR-type regulators, while for *Mesorhizobium loti* two genes were found encoding proteins with clear similarity to LuxI-like proteins and at least three LuxR-like proteins (Wisniewski-Dye & Downie, 2002). These results, even if they need further experimental confirmation, open the question whether the *B. cepacia* strain in study could have a quorum sensing response regulator protein able to use C_6 or C_7 for the activation of transcription of a specific set of genes. The finding of the *luxI/R* homologous *bviI/R* in some *Burkholderia vietnamiensis* strains (a member of the *B. cepacia* complex) in addition to the *cepI/R* (Lutter *et al.,* 2001), further validate a possible picture where more than one LuxR-type proteins are present in the quorum sensing system of *B. cepacia.*

Following the characterisation of the *B. cepacia* ATCC 25146 quorum sensing system through the present study, it was found that several phenotypes either involved directly or indirectly in pathogenesis as swarming motility, lipase, chitinase or siderophore production, were not regulated by quorum sensing. Nevertheless, the enzymatic activity polygalacturonase, previously described in this strain as an important virulence factor in onion maceration (Gonzalez *et al.,* 1997), was shown to be under the control of quorum sensing, which positively regulates the expression of this activity. This was evidenced by an attenuated in vitro maceration of onion, when the quorum sensing mutant 25416-I

was inoculated on the onion surface and also because of a reduction in the specific

activity of this enzyme in the 25416-I supernatants when compared to the wild-type

parent strain. In order to obtain a more complete picture of the quorum sensing regulon

in *B. cepacia*, a strategy was developed for the identification of quorum sensing

regulated genes. This strategy was based on the cloning of random fragments of

genomic DNA into the pSCR2 vector, screening for those that were able to activate

transcription in the presence of externally added C_8-HSL. Consequently, it was

postulated that those fragments contained promoters that can activate transcription in a

[CepR-C_8-HSL]-dependent manner. Several clones were identified, some of them

containing DNA sequences from which it was possible to deduce the presence of ORF

encoding proteins having homology to known genes. An interesting clone was P53,

which codes for a homologous of AidA (autoinducer dependent), a protein of unknown

function whose expression is quorum sensing dependent in *R. solanacearum* (Flavier *et

al.*, 1997b). However, no other known quorum sensing dependent genes were found

(e.g. *cepI* or *pehA*), meaning that the screening was not complete and a larger one would

be necessary to identify all possible loci regulated by quorum sensing. Recently, a

similar approach was described in *V. anguillarum* for the identification of genes

regulated by the LuxR homologue VanT, reporting the expression of three genes as

quorum sensing dependent (Croxatto *et al.*, 2002). In that study the expression of VanT

was induced only when L-arabinose and IPTG was provided, thus theoretically allowing

the identification of genes represed by VanT. A similar system can be used in *B.

cepacia* to complement the results obtained in the present study, since with pSCR2 it is

only possible to identify fragments of DNA whose transcription is positively activated.

On other hand, since the sequencing project of the *B. cepacia* strain J2315 is now complete, and likely soon annotated, a possible alternative approach that could further complement the results obtained in this study is the use of the cDNA microarray technology, looking for differential gene expression in the quorum sensing mutant strain.

The use of proteomics, 2D-gels combined to protein sequence, have recently demonstrated that about 5% of the proteins of *B. cepacia* H111 are under the control of quorum sensing, thus further confirming that quorum sensing is a global regulatory system (Riedel *et al.*, 2003). Nevertheless, it must still be established if quorum sensing is directly involved in the regulation of the expression of the proteins described in that study or if there are regulatory cascades present in the whole system, a fascinating field that likely will call the attention of future investigations.

The last part of this thesis refers to the identification and characterisation of the stationary phase sigma factor RpoS (σ^s) in *B. cepacia*. In *E. coli*, RpoS is a master regulator of the stress response that directs transcription of a large number of genes involved in adaptation to nutrient limiting conditions, environmental stresses and production of virulence factors (Kojic *et al.*, 1999). This is the first report of RpoS in the *B. cepacia* complex, and the second one in the *β-Proteobacteria phylum*. It is shown that, while important for the survival to heat shock and oxidative stress, it is not important in the survival to osmotic shock. It is also shown that quorum sensing is not affected by RpoS, since the accumulation of HSLs in the extracellular is comparable in both wild type and 25416-RPOS. Moreover, promoter fusion experiments show that the transcription of *rpoS* is higher in the *cepI*-null mutant than in the wild type parent strain,

while Western immunoblot analysis shows that RpoS levels are comparable in both *B. cepacia* backgrounds. These elements, together with the observation that in the WT strain *rpoS* transcription was relatively constant, while RpoS accumulates through the growth curve, suggest that in *B. cepacia* the regulation of this sigma factor takes place mainly at the post-transcriptional level. Further experimentation would be necessary to confirm this hypothesis.

Finally, Rpos has been demonstrated to be required for the production of extracellular toxins or proteases in *Y. enterocolitica, V. cholerae, P. fluorescens* and *P. aeruginosa* (Iriarte *et al.*, 1995; Sarniguet *et al.*, 1995; Suh *et al.*, 1999; Yildiz & Schoolnik, 1998). It would be interesting to verify if exist such RpoS-dependent expression of virulence factors also in *B. cepacia*.

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ANNEX

1. The *Burkholderia cepacia* ATCC 25416 quorum sensing locus (Chapter 2):

```
LOCUS          BCE422183                   2056 bp    DNA      linear    BCT 19-DEC-2001
DEFINITION     Burkholderia cepacia cepI gene and cepR gene.
ACCESSION      AJ422183
VERSION        AJ422183.1  GI:17939924
KEYWORDS       cep locus; cepI gene; CepI protein; cepR gene; CepR protein; quorum
               sensing system.
SOURCE         Burkholderia cepacia
  ORGANISM     Burkholderia cepacia
               Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales;
               Burkholderiaceae; Burkholderia; Burkholderia cepacia complex.
REFERENCE      1
  AUTHORS      Aguilar,C.A., Kojic,M. and Venturi,V.
  TITLE        The quorum sensing system of the onion pathogen type strain
               Burkholderia cepacia ATCC 25416
  JOURNAL      Unpublished
REFERENCE      2   (bases 1 to 2056)
  AUTHORS      Venturi,V.
  TITLE        Direct Submission
  JOURNAL      Submitted (12-DEC-2001) Venturi V., Bacteriology, I.C.G.E.B,
               Padriciano 99, 34012 Trieste, ITALY
FEATURES             Location/Qualifiers
     source          1..2056
                     /organism="Burkholderia cepacia"
                     /strain="ATCC 25416"
                     /db_xref="taxon:292"
     gene            complement(1..609)
                     /gene="cepI"
     CDS             complement(1..609)
                     /gene="cepI"
                     /function="autoinducer sythase"
                     /codon_start=1
                     /transl_table=11
                     /product="CepI protein"
                     /protein_id="CAD19535.1"
                     /db_xref="GI:17939925"
                     /translation="MRTFVHEEGRLPHELAADLGRYRRRVFVEQLGWALPSANESFER
                     DQFDRDDTVYVFARNAGGDMCGCARLLPTTRPYLLKSLFADLIAEDMPLPQSAAVWEL
                     SRFAATDDEGGPGNAEWAVRPMLAAVVECAAQLGARQLIGVTFASMERLFRRIGIHAH
                     RAGPPKQVDGRLVVACWIDIDPQTFAALGIEPGQAARQAIAA"
     gene            1337..2056
                     /gene="cepR"
     CDS             1337..2056
                     /gene="cepR"
                     /function="Lux R family transcriptional regulator"
                     /codon_start=1
                     /transl_table=11
                     /product="CepR protein"
                     /protein_id="CAD19536.1"
                     /db_xref="GI:17939926"
                     /translation="MELRWQDAYQQFSAAEDEQQLFQRIAAYSKRLGFEYCCYGIRVP
                     LPVSKPSVAIFDTYPDGWMAHYQAHNYIEIDSTVRDGALSTNMIVWPDVDQIEPCPLW
                     QDARDFGLSVGVAQSSWAARGAFGLLSIARHADRLTPAEINMLTLQTNWLANLSHSLM
                     SRFMVPKLSPAAGVTLTAREREVLCWTAEGKTACEIGQILSISERTVNFHVNNILEKL
                     GATNKVQAVVKAISAGLIEAP"
BASE COUNT        372 a     646 c     689 g     349 t
ORIGIN
        1 tcaggcggcg atagcttgcc gggcggcctg ccccggctcg attcctagcg cggcaaacgt
       61 ttgcggatcg atgtcgatcc agcacgcgac caccagacgc ccatctacct gcttcggcgg
      121 gcctgcgcgg tgcgcgtgta tgccgatccg gcggaacaac cgctccatgc tcgcgaacgt
      181 cacgccgatc aactgccgcg cgccgagctg cgccgcgcac tcgacgacgg ccgcgagcat
      241 cgggcgcacg gcccattcgg cgttgcccgg cccgcctccg tcgtcggtcg ccgcgaaccg
      301 ggacagctcc cagactgcgg ccgattgcgg cagcggcatg tcttcggcga tcaggtcggc
      361 gaacagcgac ttcagcagat acgggcgcgt cgtcggcagc aggcgcgcac atccgcacat
      421 gtcgccgccg gcgtttcgcg cgaacacgta gacggtatcg tcgcgatcga actggtcgcg
      481 ctcgaagctt tcgttcgccg acgggagcgc ccaaccgagc tgttcgacga acacacggcg
      541 ccgatagcgc ccgagatccg ctgcaagttc gtgtggcaac cgcccttcct cgtgaacgaa
```

```
 601 ggtccgcatg gtgtcctcgg atttgtgctt ttgtatgtgc gcattacatc gcgccgcacg
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 721 gtaacggttt cttgatcaac gggattgaaa accgggccgc ttttgcttat aaagagcgcc
 781 agcactgcgg aaattgtcag aggaatgcgg ggccagttgt cactggagcg tcggaccggc
 841 atgataggcg tcgttgtgcc cgagcagggc cgacgcgacc gctgcgggcc agtcgatgca
 901 cgcgagccgc gcggcaagcg cggcggccgt gcggaacatg ccgatgtcgg cacccgtggc
 961 gtcgacggcc atcacgttgc tgtggacgtc gccgcccgcg atcacgaacg tgcccgtcgc
1021 gcgttcgagg taccagtccc agccgaccgt cacgtaagcg acgccggcac tggccgggcg
1081 atgccattcg gtataaccgg cgaggcgtgc gtcgattgcg ctgtcgcgca gttccgcacg
1141 cagcgatgcg tcgaggccgc ttgcgacatg gtcgagcgcg agcacggcca gtgcgctttc
1201 tgacaggcgt acgtagccgt ccggggacgg gccggggacc gggtgcagca aaggtgaagt
1261 catgcgcgga atgtatcagc gtcgaacggg tgctggaacc tgacaagtat gacagcgtga
1321 cgttgtcgga gaatgaatgg aactgcgctg gcaggatgcc tatcaacaat tcagtgccgc
1381 ggaagacgag cagcagctct tccagcggat tgccgcctat tccaaacggc tgggattcga
1441 atactgctgt tatggcattc gcgtgccgct gccggtatcg aagccgtcgg tagccatctt
1501 cgatacctat cccgacggct ggatggcgca ctatcaggcg cacaactaca tcgagatcga
1561 ttcgacggtt cgcgacggcg cgctcagcac caacatgatc gtctggccgg acgtcgacca
1621 gatcgaaccg tgcccgctgt ggcaggacgc gcgggatttc gggctgtcgg tgggcgtcgc
1681 gcaatcgagc tgggcggccc gcggcgcgtt cggcctgctg agcatcgcac gccatgcgga
1741 ccgcctgacg ccggccgaaa tcaacatgct gacgctgcag acgaactggc tcgcgaacct
1801 gtcgcattcg ctgatgagcc gcttcatggt gccgaagctg tcgccggcgg cgggcgtcac
1861 gctgaccgcg cgcgagcgcg aggtgctgtg ctggacggcc gagggcaaga ccgcgtgcga
1921 aatcggccag atcctcagca tctcggagcg aacggtcaac ttccacgtca acaacatcct
1981 cgagaagctg ggtgcgacga acaaggtcca ggcggtcgtc aaggcaatct cggccggact
2041 gatcgaggcg ccctga
```

2. Quorum sensing-controlled DNA fragments (Chapter 3):

>P1
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>P15
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TGGCCGAATATGGGGAATAAAAATGAAAGACCACAGCACATCACGGTTCGAGAACCGGATTAAAAACATATTGCAGCTTATCCGGGAGA
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>P25
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G

>P38
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>P53
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>P55
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>P56
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>P57
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>P59
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>P67
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>P68
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>P69
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>P79
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>P80
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>P81
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>P85
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>P88
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>P91
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>P94
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TCGTGGCTGAAATCCATTTTAACTGAG

>P96
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GGCGAATGGATCGGTGCGAGCGAACGCGAAACGGCGCCGGTGATCAACCCCGCCACGCAAATCGAGATCGGGCGCGTCCCGCTGGCGAC
GGCAGCCGATCTGGATCGCGCCCTGCAGGCCACCGCACCGGCTTTTGACGTCTGGCGCAACACCGTGCCTGCCGAACGCGCGCGCATTC
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>P103
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>P105
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GCAG

>P110
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CGCGCAGGCGCACTACGGCAAGGATATGCAGAACGGGATCGTGCTCG

>P111
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TGCCTGTGATTGCCGTAGGGCGGGCAGGTGCGCGGTCTCGAGGACCAGGATCGCGCGGAACTCGGCATTCCCGACGAAGCGGCGATCGT
TGCGCGTTATTGCGAGCTGCGCTGGCATAGATCCGATCCGCGACTGGCATTATGTACCTCGCATTCAGTTTCTTCCGGCTCGCGCAGAT
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>P114
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>P121
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>P122
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>P130
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>P135
CTGACGATCGGCTGATCGGGCGGCAGGCCGCGGGTGGCGCCGGCATGCGCTGCCCGACAGGAGGAAACGGCGCGCGGAGCGATCCGCGC
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G

3. The *Burkholderia cepacia* ATCC 25416 *rpoS* gene (Chapter 4):

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LOCUS        BCE457984                1089 bp    DNA      linear    BCT 23-APR-2002
DEFINITION   Burkholderia cepacia rpoS gene for RNA polymerase sigma factor.
ACCESSION    AJ457984
VERSION      AJ457984.1  GI:20330083
KEYWORDS     RNA polymerase sigma factor; rpoS gene.
SOURCE       Burkholderia cepacia
  ORGANISM   Burkholderia cepacia
             Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales;
             Burkholderiaceae; Burkholderia; Burkholderia cepacia complex.
REFERENCE    1
  AUTHORS    Aguilar,C.A., Bertani,I., Kojic,M. and Venturi,V.
  TITLE      The quorum sensing system and the stationary phase sigma factor
             (rpoS) of the onion pathogen Burkholderia cepacia Gv.I type strain
             ATCC 25416
  JOURNAL    Unpublished
REFERENCE    2   (bases 1 to 1089)
  AUTHORS    Venturi,V.
  TITLE      Direct Submission
  JOURNAL    Submitted (22-APR-2002) Venturi V., Bacteriology, ICGEB, Area
             Science Park, Padriciano 99, Trieste/34012, ITALY
FEATURES             Location/Qualifiers
     source          1..1089
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                     /strain="ATCC 25416"
                     /db_xref="taxon:292"
     gene            1..1089
                     /gene="rpoS"
     CDS             1..1089
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                     /codon_start=1
                     /evidence=experimental
                     /transl_table=11
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                     /translation="MPKSKRHEPQAESEKISRATQASVGRAGASTDDEDDNADNERDL
                     EARDAEADGGDDEREGRAEASPDVDDFRTLLQAELTADTIQHYLNRISVKPLLTVEEE
                     QRYSRLAKAGEFEARQVMIERNLRLVVSIAKGYLNRGVPLLDLIEEGNLGLMHAIEKF
                     DPTRGFRFSTYATWWIRQSIERAIMNQARTVRLPVHVIRELNQVLRAKRHLEKNSMST
                     GEAAERREASIDDIAYLTGKTAEEVTDILALNEHTASLDAPLDLDPASSLLDLLPDDQ
                     SQSPDAEVQHRELETLTRAWLSRLSDKHRHVIERRFGLNHIEPATLEELADEMGLTRE
                     RVRQIQQEALVRLKRFFASNGVRKDAVL"
BASE COUNT        225 a      365 c       352 g       147 t
ORIGIN
        1 atgccgaaat cgaagcgcca cgagccgcaa gccgagtctg agaagatcag tcgtgccacg
       61 caagcatcgg tggggcgagc aggtgcttcg acggacgacg aagacgacaa cgcggataac
      121 gaacgcgatc tcgaggcgcg cgacgccgag gcggacggtg gcgacgacga gcgcgaaggc
      181 cgggccgagg cgtcgcccga cgtcgacgat ttccgcacgc tgctgcaggc cgaactgacg
      241 gccgacacga tccagcatta cctgaaccgc atcagcgtga agccgctgct caccgtcgag
      301 gaggagcagc gctattcgcg tctggccaag gccggtgaat tcgaggcgcg gcaggtgatg
      361 atcgagcgca acctgcgcct cgtcgtcagc atcgcgaagg gatacctgaa ccgtggcgtg
      421 ccactgctcg acctgatcga ggaaggcaac ctcggcctga tgcatgcgat cgaaaaattc
      481 gacccgacgc gcggcttccg tttttcgacc tacgcgacgt ggtggatccg ccagagcatc
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     1021 caggaagcgc tggtgcgcct caagcggttt ttcgcctcca acggcgtgcg caaggacgcc
     1081 gttctgtaa
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