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Quorum-Sensing System and Stationary-Phase Sigma Factor (*rpoS*) of the Onion Pathogen *Burkholderia cepacia* Genomovar I Type Strain, ATCC 25416

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Bacterial strains belonging to *Burkholderia cepacia* can be human opportunistic pathogens, plant pathogens, and plant growth promoting and have remarkable catabolic activity. *B. cepacia* consists of several genomovars comprising what is now known as the *B. cepacia* complex. Here we report the quorum-sensing system of a genomovar I onion rot type strain ATCC 25416. Quorum sensing is a cell-density-dependent regulatory response which involves the production of *N*-acyl homoserine lactone (HSL) signal molecules. The *cep* locus has been inactivated in the chromosome, and it has been shown that CepI is responsible for the biosynthesis of an *N*-hexanoyl HSL (C_6-HSL) and an *N*-octanoyl HSL (C_8-HSL) and that the *cep* locus regulates protease production as well as onion pathogenicity via the expression of a secreted polygalacturonase. A *cep-lacZ*-based sensor plasmid has been constructed and used to demonstrate that CepR responded to C_6-HSL with only 15% of the molar efficiency of C_8-HSL, that a *cepR* knockout mutant synthesized 70% less HSLs, and that CepR responded best towards long-chain HSLs. In addition, we also report the cloning and characterization of the stationary-phase sigma factor gene *rpoS* of *B. cepacia* ATCC 25416. It was established that quorum sensing in *B. cepacia* has a negative effect on *rpoS* expression as determined by using an *rpoS-lacZ* transcriptional fusion; on the other hand, *rpoS*-null mutants displayed no difference in the accumulation of HSL signal molecules.

Burkholderia cepacia was first described as a potent phytopathogen responsible for the bacterial rot of onions (5). Bacterial strains of *B. cepacia* are recognized as major opportunistic pathogens in patients with fibrocystic lung disease (20). *B. cepacia* strains can degrade complex herbicides and pesticides (10) and also behave as plant growth-promoting rhizobacteria by suppressing soil-borne plant pathogens (3, 6, 35). *B. cepacia* consists of groups or subpopulations termed genomovars (49), 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 at least nine genomic species originally referred to as genomovars I to IX (7, 48, 49). Taxonomic studies have shown that this 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 (15). In gram-negative bacteria, *N*-acyl homoserine lactone (HSL) autoinducers appear to be the most commonly used signaling molecules; in most cases they are produced by an autoinducer synthase protein belonging to the LuxI family. A transcriptional activator belonging to the LuxR family forms a complex with the cognate autoinducer at high threshold levels to induce transcriptional activation of target genes. 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, and consequently also the HSL concentration, reaches a quorum, thereby activating the LuxR type protein, which then elicits the desired cellular response. Quorum sensing has been implicated in the regulation of biofilm formation, plasmid transfer, and motility and in several virulence factors (53).

Another important regulatory component involved in regulation in response to high cell density is the stationary-phase sigma factor RpoS (25, 30). RpoS is an alternative sigma factor which directs transcription of a large number of genes involved in adaptation to nutrient-limiting conditions and several environmental stresses as well as inducing the production of virulence factors (30, 47). In *Escherichia coli* and *Pseudomonas* spp., RpoS levels are induced as bacterial cultures enter the stationary phase (14, 26, 27), and it has also been reported that quorum sensing and RpoS cross-regulate their gene expression in *Pseudomonas aeruginosa* (28, 51).

The quorum-sensing system of a *B. cepacia* genomovar III cystic fibrosis respiratory isolate has been identified and characterized and consists of *cepI* and *cepR* genes. This bacterial isolate, designated K56-2, synthesizes an *N*-octanoyl HSL (C_8-HSL) and an *N*-hexanoyl HSL (C_6-HSL) (29, 32). The CepI/R quorum-sensing system of *B. cepacia* K56-2 has been implicated in the negative regulation of the siderophore ornibactin and in the positive regulation of a secreted protease. Similarly, the CepI/R quorum-sensing system from another *B. cepacia* genomovar III cystic fibrosis respiratory isolate, designated H111, has been recently identified and characterized and shown to be involved in regulating biofilm formation and swarming motility (23). In addition, quorum-sensing systems are present and conserved among the heterogeneous *B. cepacia* complex (19). The stationary-phase sigma factor RpoS, on

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TABLE 1. Plasmids used in this study

Plasmid	Description	Source or reference
pBluescript KS	Ap^r, Co1E1 replicon	Stratagene
pUC18	Ap^r, Co1E1 replicon	54
pMP190	Cm^r, IncQ, promoter probe vector	44
pUC4K	Ap^r, pBR322 replicon	Amersham Pharmacia
pMP77	Cm^r, IncQ, promoter probe vector	44
pRK2013	Km^r, Tra^+ Mob^+, Co1E1 replicon	12
pPH1J1	Gm^r IncP1	1
pSUP2021	Ap^r Cm^r Tc^r Km^r pBR325 replicon, broad-host-range vector	43
pLAFR3	Tc^r, broad-host-range cloning vector, IncP1	45
pQF50	Ap^r, broad-host-range vector, pRO1600 replicon	11
pLIR5	Tc^r, pLAFR3 containing *cepIR* locus in a 9-kbp *Eco*RI fragment	This study
pBIR	Ap^r, pBluescript KS containing *cep1R* locus in a 9-kbp *Eco*RI fragment	This study
pCQS1	Tc^r, pLAFR3 containing *B. cepacia* DNA	This study
pCQS2	Tc^r, pLAFR3 containing *B. cepacia* DNA	This study
pCQS3	Tc^r, pLAFR3 containing *B. cepacia* DNA	This study
pLIR::Tn*5*3	Tc^r, Km^r, pLIR5 with a Tn*5* insertion in *cepR*	This study
pLCIKm	Tc^r, Km^r, pLIR5 with a Km^r 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 *Pst*I fragment	This study
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 a 5-kbp *Sma*I fragment from pCOSRPOS-2C	This study
pRBS-3	Ap^r, pBluescript KS containing a 1-kbp *Pst*I fragment from pCOSRPOS-2C	This study
pRBS-3Km	Ap^r Km^r, pRBS-3 with a Km^r cassette in the *rpoS* gene	This study
pCOSRPOS-2CKm	Tc^r Ap^r Km^r, pCOSRPOS-2C with a Km^r cassette in the *rpoS* gene	This study
pRPR2	Cm^r, *PrpoS-lacZ* fusion in pMP190 promoter probe vector	This study

the other hand, has to our knowledge not been reported in any bacterial strains belonging to the *B. cepacia* complex.

In this study we report the identification and characterization of the quorum-sensing system of the onion pathogen *B. cepacia* type strain ATCC 25416 which belongs to genomovar I. This strain has been isolated from a rotten onion, and it has a genome of 8.1 Mb composed of four circular replicons of 3.65 Mb, 3.17 Mb, 1.07 Mb and 200 Kbp (39). We have constructed a *cepR*-P*cepI-lacZ* reporter plasmid and shown (i) that a *cepR* knockout mutant results in a 70% decrease in HSL production, CepR responded to C_6-HSL with only 15% the molar efficiency of C_8-HSL, and CepR responds best to long chain HSL autoinducers and (ii) that *cepI* and *cepR* knockout mutants are attenuated in onion pathogenicity. The *rpoS* gene of *B. cepacia* ATCC 25416 has also been identified and characterized. *B. cepacia* RpoS did not display very high identity in its primary structure to RpoS belonging to the γ-*Proteobacteria*. RpoS was not involved in HSL production, and quorum sensing had a negative effect on *rpoS* expression.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The plasmids used in this study are listed in Table 1. *B. cepacia* ATCC 25416 (or LMG 1222) is an isolate from a rotten onion, and it was routinely grown in Luria-Bertani (LB) broth (40), M9 minimal medium (40), or M9GP (18) at 30°C. *Chromobacterium violaceum* CVO26 is a double mini-Tn*5* mutant derived from ATCC 31532. This mutant is nonpigmented, and production of the purple pigment can be induced by providing exogenous HSL inducer molecules (34). The *E. coli* strains used in this study included HB101 (40), DH5α (21), and HB101::Tn*5* (33) and were grown in LB medium (36) at 37°C. Antibiotics were added as required at the following final concentrations: tetracycline, 10 (for *E. coli*), 20 (for *C. violaceum*), or 300 (for *B. cepacia*) μg/ml; gentamicin, 10 (for *E. coli*) or 300 (for *B. cepacia*) μg/ml; ampicillin, 100 μg/ml (for *E. coli*); kanamycin, 50 (for *E. coli*) or 300 (for *B. cepacia*) μg/ml; and streptomycin, 100 μg/ml (for *C. violaceum*).

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 hybridization, and transformation of *E. coli* were performed as described previously (40). Analytical amounts of plasmids were isolated as described previously (4), whereas preparative amounts were purified with Qiagen columns. Total DNA from *B. cepacia* was isolated by the sarcosyl-pronase lysis method (2). Triparental matings from *E. coli* to *B. cepacia* were performed with the helper strain *E. coli*(pRK2013) (12). A genomic cosmid library of *B. cepacia* ATCC 25416 was constructed by using a Gigapack III XL packaging extract kit (Stratagene, La Jolla, Calif.) and following the instructions provided by the supplier. Briefly, genomic DNA was partially digested with *Eco*RI and ligated into pLAFR3. Concatemers were then packaged into lambda phages and used to infect *E. coli* HB101 cells.

Cloning of quorum-sensing genes of *B. cepacia* ATCC 25416. About 4×10^9 cells (each) of *E. coli* HB101 harboring the *B. cepacia* ATCC 25416 cosmid library and *E. coli*(pRK2103) and 2×10^8 cells of *C. violaceum* CVO26 were mixed. The suspension was applied to a 0.45-μm-pore-size membrane filter (Millipore Corp.) on an LB plate. After overnight incubation at 30°C, the 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 mini-Tn*5* present on the chromosome. Tetracycline will select transconjugants that have received the pLAFR3-based cosmid clone. These plates were incubated for 48 h at 30°C, and transconjugants that turned purple were further assayed. Three cosmids (pCQS1 to pCQS3) from the cosmid library could restore purple pigmentation, and they only shared a 9-kb *Eco*RI common insert. Further subcloning experiments confirmed that the 9-kb *Eco*RI fragment cloned in pLAFR3 (creating pLIR5) could restore pigmentation on strain CVO26. Thin-layer chromatographic analysis with extracts from spent supernatants of strain CVO26(pLIR5) showed that C_6- and C_8-HSL were now synthesized.

Transposon Tn*5* mutagenesis. Transposon Tn*5* insertions within recombinant plasmid pLIR5 were obtained as described previously (33), with *E. coli* HB101::Tn*5* as the source of the transposon. *E. coli* HB101 cells containing Tn*5* insertions within plasmid pLIR5 were identified by purifying plasmid DNA from HB101::Tn*5*(pLIR5), using it to transform *E. coli* DH5α, and selecting for plasmids having tetracycline and kanamycin resistance. These recombinant plasmids in DH5α were delivered by triparental conjugation to *C. violaceum* CVO26

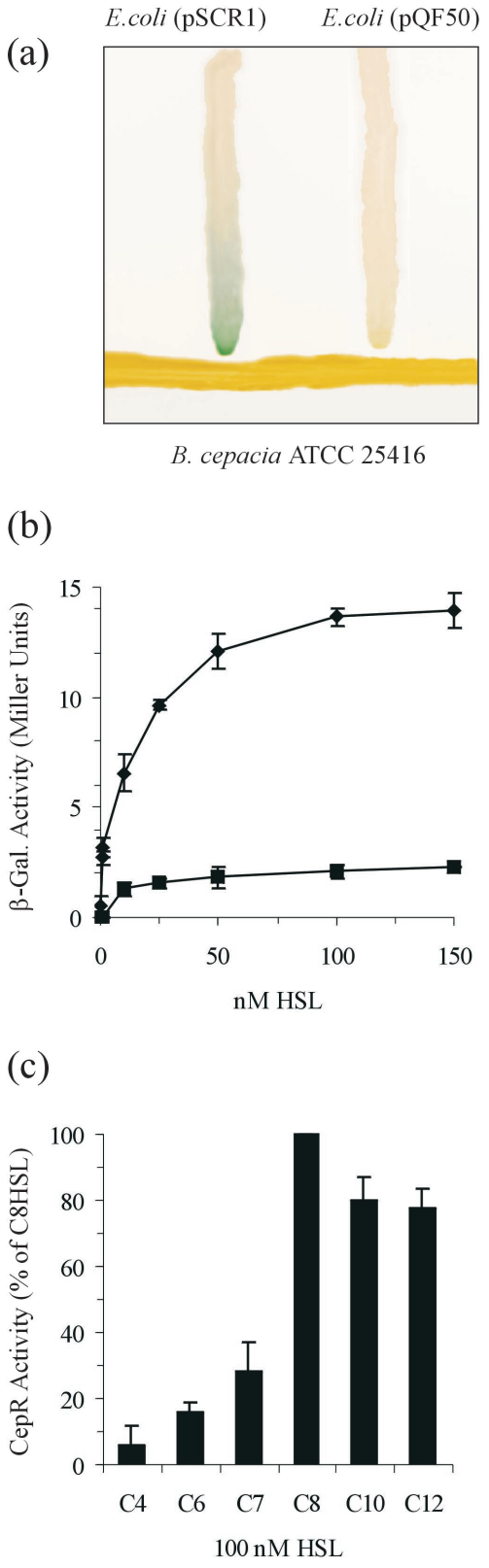

FIG. 1. (a) Activation of bacterial sensor strain in cross-streak experiments. The sensor strain *E. coli* DH5α(pSCR1) was cross streaked on LB agar plates (containing ampicillin and X-Gal) with *B. cepacia* ATCC 25416 used as a tester strain. (b) HSL bioassay with *E. coli* DH5α(pSCR1). DH5α(pSCR1) was grown in the presence of various concentrations of either C_6-HSL (■) or C_8-HSL (♦), and β-galactosidase activities were determined after 6 h. The values were determined with LB medium, the means of triplicate experiments are given, and the standard deviations are shown. (c) CepR-HSL response. *E. coli* DH5α(pSCR1) was grown for 6 h in the presence of a 100 nM concentration of either C_4-, C_6-, C_8-, C_{10}-, or C_{12}-HSL, and β-galactosidase activities were determined. The values were determined with LB medium, the means of triplicate experiments are given, and the standard deviations are shown. The values are expressed as percentages of the activity determined with C_8-HSL.

as described above. Transconjugants of strain CVO26(pLIR5::Tn*5*) lacking the expression of violacein (i.e., those which remained white) were purified, and the position of Tn*5* was mapped within the 9-kb *Eco*RI insert. Three insertions were located in the *cepR* gene (data not shown).

Construction of a *B. cepacia* ATCC 25416 *cepI* and *cepR* knockout mutants. Plasmid pLCIKm was constructed as follows. A 3.5-kb *Hin*dIII DNA fragment from pSUP2021containing the Km^r gene of transposon Tn*5* was cloned into the corresponding site in *cepI* in plasmid pLIR5. Plasmid pLIR::Tn*53* (which contain a Tn*5* insertion in the *cepR* gene harbored in pLAFR3) and plasmid pLCIKm (which contains a kanamycin resistance gene cloned in the *cepI* gene) were homogenized with the corresponding target regions of the genome of *B. cepacia* ATCC 25416 by a marker-exchange procedure (9). Plasmid pPH1JI was used as the incoming IncP1-incompatible plasmid, and selections were made on LB plates containing kanamycin and gentamicin. This generated two genomic mutants, designated *B. cepacia* 25416-I and *B. cepacia* 25416-R, which harbored a Km^r 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 (data not shown).

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 *Bam*HI-*Hin*dIII 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 (11), 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. The β-galactosidase activity was determined as described previously (34, 43).

Purification, detection, and visualization of autoinducer molecules (HSLs). The purification, detection, and visualization of HSL inducer molecules from culture supernatants were performed essentially as described previously (25, 34). Synthetic HSLs (C_4-HSL, C_6-HSL, C_7-HSL, C_8-HSL, C_{10}-HSL, and C_{12}-HSL) were purchased from Fluka Chemie AG (Buchs, Switzerland). For quantification of CepR activity, overnight *E. coli* DH5α(pSCR1) cultures were normalized to an optical density at 600 nm (OD_{600}) of 0.1 in a volume of 20 ml of LB containing the desired HSL at the desired concentration. Cultures were then grown with agitation at 37°C for 6 h, and β-galactosidase activities were determined. The presence of HSL was also detected on solid media by growing *E. coli*(pSCR1) on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and ampicillin media in close proximity to the tester strain. The presence of HSL was observed when *E. coli*(pSCR1) turned blue (Fig. 1a).

Cloning of the *rpoS* gene of *B. cepacia* ATCC 25416. The *rpoS* DNA sequences from cloned and characterized *Pseudomonas* spp. (25) were compared with the genome sequence of *B. cepacia* genomovar III clinical isolate strain J2315 (www.sanger.ac.uk/Projects/B_cepacia/). The putative *rpoS* of *B. cepacia* J2315 was localized in contig Bcep1047b02.g1c at positions 10602 to 11690. Two synthetic primers were designed from conserved regions (rpoS-P3, 5′-CTGCTCGACCT GATCGA-3′, and rpos-P6, 5′-AGGCTGCTCGCGGGATC-3′) and used in a PCR amplification reaction. This resulted in the amplification of a 300-bp fragment, which was cloned in pUC18, yielding pRPC-1. This fragment was then used as a probe against the cosmid library of *B. cepacia* ATCC 25416. A cosmid designated pCOSRPOS-2C was identified, and the *rpoS* gene was localized within a 5.5-kb *Sma*I fragment and in part in a 1-kb *Pst*I fragment (see Fig. 4). These were cloned in the corresponding sites in pBluescript KS, yielding pRBS-2 and pRBS-3, respectively.

Construction of a *B. cepacia* ATCC 25416 *rpoS* knockout mutant. The kanamycin-resistant gene from pUC4K as a *Bam*HI fragment was cloned in the corresponding site in the pRBS-3, creating pRBS-3Km and resulting in an interruption of the *rpoS* gene of *B. cepacia* ATCC 25416 (see Fig. 4). This *rpoS*-interrupted locus in pRBS-3Km was transferred by homologous recombination to the *rpoS* gene harbored in pCOSRPOS-2C in the following way: pRBS-3Km

was used to transform DH5α(pCOSRPOS-2C), the resulting DH5α (pCOSRPOS-2C)(pRBS-3Km) was grown overnight, and the culture was used in a triparental conjugation into *Pseudomonas putida* WCS358 (16) with *E. coli*(pRK2013) as a helper. After appropriate selection, pCOSRPOS-2CKm was selected. Transfer of the Km^r cassette by double-crossover homologous recombination from pRBS-3Km to pCOSRPOS-2 was verified by restriction and Southern analysis. The plasmid pCOSRPOS-2CKm was then used in a marker-exchange technique, as described above, in order to introduce site-specific insertion mutations into the *rpoS* gene of *B. cepacia* ATCC 25416. The fidelity of the marker-exchange event in the *B. cepacia rpoS*::Km^r mutant was confirmed by Southern analysis (data not shown). This mutant was designated *B. cepacia* 25416-RPOS.

Exoenzyme assays, siderophore production, in vitro maceration of onion tissue, and stress response assays. Proteolytic, lipolytic, and chitinolytic activity were determined on the appropriate indicator plates (23) and also determined spectrophotometrically (29). Polygalacturonase activity was determined as previously described (18). In order to determine the ability to macerate onion tissue, overnight LB cultures were normalized at an OD_{600} of 1, and 100 μl of culture was inoculated onto the surface of onion tissue prepared as described previously (52). Clean, disease-free onions (*Allium cepa*) were wiped with 90% (vol/vol) alcohol before being cut aseptically into slices. Onion slices were placed into sterile petri dishes, and nicks of approximately 2 mm were made in the tissue surface. Plates were incubated at 30°C, and readings were taken after 48 h of incubation. The measurement of cell viability and ability to survive heat stress and osmotic stress and sensitivity to hydrogen peroxide were determined as described previously (25).

DNA sequence determination and analysis. The DNA sequence of the *cepI*/*R* locus was determined by using pBIR as template. The DNA sequence of the *rpoS* gene was determined by using plasmids pRBS-2 and pRBS-3 as templates. Nucleotide sequences were determined by the dideoxy chain-termination method (41) by using [^{35}S]dATPαS for labeling and 7-deaza-dGTP (Pharmacia) instead of dGTP.

Nucleotide accession number. The sequence of *cepI-cepR* locus and the *rpoS* gene have been deposited in the GenBank/EMBL/DDBJ database under accession numbers AJ422183 and AJ457984, respectively.

RESULTS

The quorum-sensing system of *B. cepacia* ATCC 25416. The quorum-sensing systems of two *B. cepacia* clinically isolated strains (genomovar III) have been associated with roles in siderophore production and ability to form biofilms (23, 29). In addition, it has been reported that many strains belonging to the *B. cepacia* complex produce HSL molecules with an aliphatic chain composed of C_6 and C_8 (19). The quorum-sensing system of *B. cepacia* genomovar I ATCC 25416 produces both C_8- and C_6-HSL, and *cepI* and *cepR* genes have been previously cloned by PCR amplification (19). In this study, we have cloned and characterized the complete genomic locus coding for CepIR in *B. cepacia* ATCC 25416 (accession number AJ422183). In order to study this quorum-sensing system, we constructed a sensor plasmid pSCR1 which is made of components of the *cep* quorum-sensing system of strain ATCC 25416. This sensor plasmid contains the promoter of *cepI* transcriptionally fused to a promoterless *lacZ* reporter gene together with the *cepR* gene and intergenic region in plasmid pQF50. It was observed that on solid media containing X-Gal, pSCR1 harbored in *E. coli* can conveniently be used to detect the production of HSL when streaked in close proximity to a tester strain (Fig. 1a).

Since *B. cepacia* ATCC 25416, like many other strains of the *B. cepacia* complex (19), produced C_6- and C_8-HSL molecules, we tested the activity of these two molecules by using plasmid pSCR1. CepR responded to C_6-HSL with only 15% of the molar efficiency of C_8-HSL; the plasmid detected the latter molecule at a concentration of less than 10 nM and reached its highest β-galactosidase activity when a 100 nM concentration of the molecule was present. Higher concentrations did not result in higher reporter enzyme activity (Fig. 1b). The C_6-HSL, on the other hand, did not result in a good response with CepR (Fig. 1b), even at very high concentrations as high as 1,000 nM (data not shown).

It was of interest to determine the response of the CepR-based sensor plasmid with respect to different HSL molecules. This was determined by measuring the β-galactosidase activities of *E. coli*(pSCR1) exposed to 100 nM concentrations of various HSL molecules. It was decided to use 100 nM since it was the minimum concentration of C_8-HSL (the major molecule produced by *B. cepacia*) necessary to achieve a good response (see Fig. 3a). As expected, the constructed biosensor responded best to C_8-HSL when compared to C_4-, C_6-, C_7-, C_{10}-, and C_{12}-HSL. In Fig. 1c, the percentage of the CepR response to C_8-HSL is presented, and as shown, CepR displayed a good response to long-chain signal molecules and very poor one to short-chain signal molecules.

The *cepI* and *cepR* genes were insertionally inactivated independently, creating two genomic mutants, 25416-I and 25416-R, respectively. The 25416-I mutant no longer produced the C_6- and C_8-HSL (data not shown). The 25416-R synthesizes approximately 30% of the signal molecules produced by the wild-type parent strain (data not shown). Since the detecting CepR-based plasmid is not specific to C_6-HSL, this value refers mainly to C_8-HSL. Furthermore, on the basis of the β-galactosidase activity obtained, it was estimated that a culture of *B. cepacia* ATCC 25416 at an OD_{600} of 1 contains an approximately 100 nM concentration of C_8-HSL signal molecules. Both 25416-I and 25416-R could be complemented for HSL production when plasmid pMPIR, containing the *cepI* and *cepR* genes, was introduced by triparental conjugation (data not shown).

CepIR regulates onion pathogenicity in *B. cepacia* ATCC 25416. It was observed that 25416-I and 25416-R displayed similar lipolyitc and chitinolytic activities and siderophore production when compared to the wild-type parent strain, but the proteolytic activity, on the other hand, was significantly lower in the two mutants. *B. cepacia* ATCC 25416 was originally isolated as a pathogen responsible for the rot of onions (5), and it was therefore of interest to check if quorum sensing was related to rot. Experiments with in vitro maceration of the onion *A. cepa* resulted in both 25416-I and 25416-R mutants having attenuated maceration compared to the wild type; introduction in *trans* of pMPIR carrying the *cepI*/*R* locus resulted in more-severe onion pathogenicity than in the wild type (Fig. 2). The attenuated maceration in the onion could be attributed to the decrease in production of extracellular enzymes involved in the onion maceration. One such example could be polygalacturonase, which has been identified and characterized in this strain and implicated in onion disease development (18). We determined polygalacturonase activity in spent culture supernatants of ATCC 25416 and mutant 25416-I. The enzyme activity in inducing conditions was reduced 40% in 25416-I, whereas in the complemented mutant, 25416-I(pMPIR), the activity was 140% of the wild-type levels (Table 2). Quorum sensing was therefore implicated in onion pathogenicity in this strain, and this is at least in part due to the hydrolytic secreted enzyme polygalacturonase. Similar attenuation and comple-

FIG. 2. Role of quorum sensing in onion rot. One hundred microliters of a culture of the indicated strain with an OD_{600} of 1 was inoculated on the right half of a sterile onion as described in the text. This picture was taken after 48 h of incubation at 30°C.

mentation levels of enzyme activity were obtained with 25416-R (data not shown).

Stationary-phase *rpoS* gene of *B. cepacia* ATCC 25416. The nucleotide sequence of the putative *rpoS* gene of strain ATCC 25416 was determined and consists of 1,089 nucleotides (Fig. 3a) (accession number AJ457984). The RpoS protein consists of 362 amino acids with a molecular mass of 41 kDa. Interestingly, it displayed the highest amino acid identity with other putative RpoS proteins of the *Burkholderia* genus (98% with *B. cepacia* genomovar III J2315, >88% with *Burkholderia fungorum*, and 93% with *Burkholderia pseudomallei* and *Burkholderia mallei*) and 74% identity with the experimentally determined RpoS of *Ralstonia solanacearum*, whereas it displayed approximately 50% identity with the sigma factors of γ-*Proteobacteria* (e.g., *P. aeruginosa*, *E. coli*, and *Vibrio cholerae*). In order to investigate the physiological role that *rpoS* played in *B. cepacia*, we insertionally inactivated *rpoS* in strain ATCC 25416, yielding an *rpoS*::Km^r knockout mutant called *B. cepacia* 25416-RPOS. We tested the response of 25416-RPOS to various environmental conditions, as the RpoS sigma factor is known to confer cross-protection against several stresses in other gram-negative bacteria (17, 38, 47). We tested the resistance of the parent strain and 25416-RPOS against heat shock, increased osmolarity, and hydrogen peroxide. Strain 25416-RPOS was more sensitive to heat shock and oxidative stress than the wild-type strain; however, there was no significant difference when cells were exposed to an increase in osmotic pressure (Fig. 3b, c, and d)

TABLE 2. Polygalacturonase activity from spent culture supernatants of *B. cepacia* ATCC 2516 and 25416-I

Strain	Polygalacturonase activity (U/mg)[a]	% of ATCC 25416 polygalacturonase activity
ATCC 25416	2,300	100
25416-I	1,380	60
25416-I(pMPIR)	3,220	140

[a] Cultures were grown for 16 h in M9GP medium and had similar OD_{650}s (18). For growth media and enzyme activity, see Materials and Methods. The mean values of triplicate experiments are given, and standard deviations were ±5%.

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 the stationary phase.

In order to determine whether *rpoS* influenced the accumulation of HSL signal molecules, we quantified HSL production in 25416-RPOS and compared it to the values obtained with the wild type strain. Signal molecules were extracted from spent supernatants of the wild-type and mutant strains at different growth stages, and the extracts were assayed with *E. coli*(pSCR1). Figure 4a depicts the results showing that the *B. cepacia* mutant lacking RpoS synthesizes at different growth stages approximately the same amount of signal molecules produced by the wild-type parent strain, 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, creating pRPR2. *rpoS* promoter activity was then assayed at different growth stages in the 25416-I mutant and compared to the promoter activity obtained with the wild-type strain. As depicted in Fig. 4b, *rpoS* expression in the wild-type strain was relatively constant during the early, exponential, and stationary phases. It was then determined that in 25416-I, *rpoS* expression increased two- to threefold in all growth stages; this increase in activity was retained even when a 100 or 1,000 nM concentration of C_8-HSL was added to the growth media (Fig. 4b). A similar increase in *rpoS* promoter activity was also observed in the *cepR* mutant 25416-R (data not shown). In view of the fact that *rpoS* expression increased in the quorum-sensing mutants, the resistance against heat shock and hydrogen peroxide was determined in mutant 25416-I. It was observed, however, that the resistance to the two stresses was comparable to what was observed with the wild-type strain (data not shown).

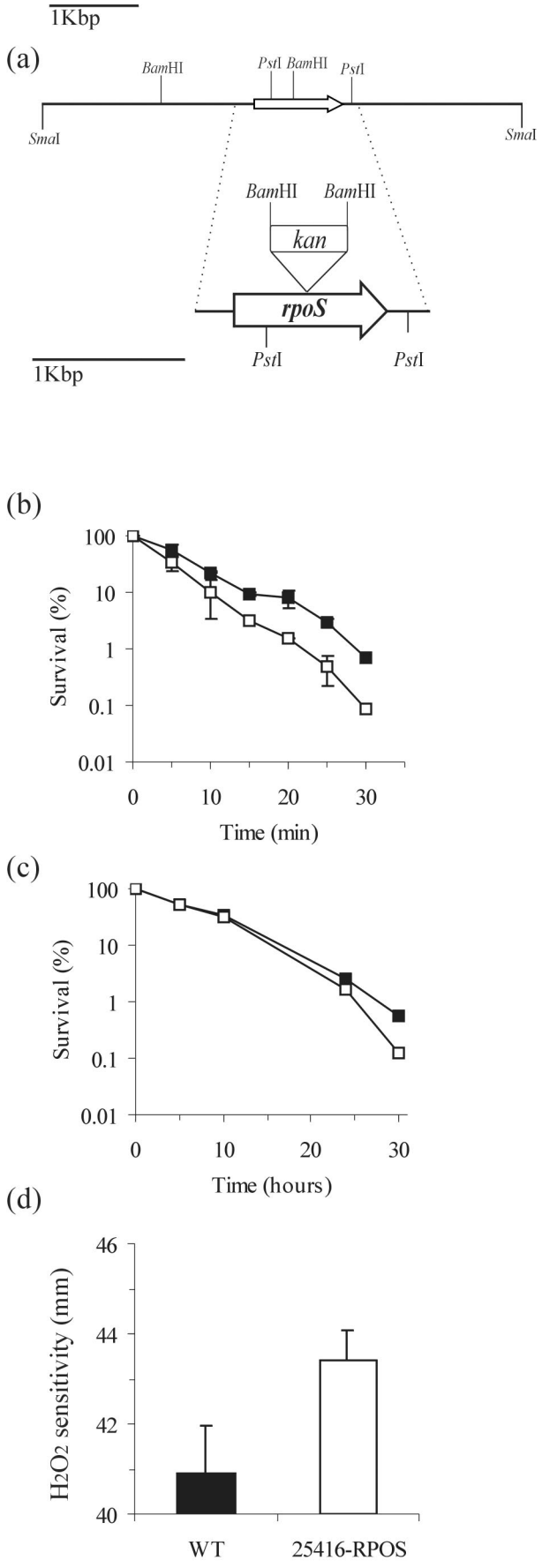

FIG. 3. (a) Map of the 5.5-kbp *Sma*I DNA fragment from *B. cepacia* ATCC 25416 isolated in this study. Shown are several enzyme restriction sites and the location of the *rpoS* gene within this fragment. Also shown is the position where a Km^r-containing *Bam*HI fragment derived from pUC4K was cloned in the corresponding site of the *rpoS* gene to create pLCIKm. (b, c, and d) Effect of *rpoS* on stress responses of *B. cepacia* ATCC 25416. (b) Response to heat shock (50°C). Viability is expressed as a percentage of the number of CFU at time zero. (c) Response to osmotic shock (2 M NaCl). Viability is expressed as a percentage of the number of CFU at time zero. (d) Effect of *rpoS* mutation on oxidative stress. The sensitivity to H_2O_2 was measured on cells grown for 16 h in LB at 30°C. The zones of inhibition were measured in millimeters. Filled symbols, WT strain; open symbols, *B. cepacia* 25416-RPOS strain.

DISCUSSION

In this study we identified and characterized the genetic determinants coding for the quorum-sensing system and for the stationary-phase sigma factor RpoS in *B. cepacia* genomovar I type strain ATCC 25416. To our knowledge, this is the first report of the *rpoS* gene in the *B. cepacia* complex, other genes have been sequenced and are highly identical (83 to 98%, see above). This is only the second *rpoS* gene characterized from a β-*Proteobacteria*; interestingly, RpoS proteins belonging to β-*Proteobacteria* are not so closely related to the RpoS proteins of γ-*Proteobacteria* (22). These two loci, *cepI*/*R* and *rpoS*, involved in stationary-phase gene expression were inactivated and studied.

B. cepacia ATCC 25416, like other members of the *B. cepacia* complex, synthesizes C_6- and C_8-HSL molecules (19). The synthesis of both appears to require the CepI protein reported here, since a *cepI* knockout mutant did not synthesize either (data not shown). It is possible that the *cepI* synthase directs the synthesis of C_8-HSL and lower traces of C_6-HSL by recognizing two different acyl-acyl carrier proteins (37). It was observed that our CepR-based biosensor in *E. coli* has a stronger response to long-chain HSLs than to short-chain HSLs, and it displays the best response to the C_8-HSL, the major molecule synthesized by *B. cepacia*. It must be stressed, however, that these results will not necessarily reflect what the in vivo situation is in *B. cepacia*, since it has been reported that for the LuxR homologue CarR of *Erwinia carotovora*, the protein concentration as well as the bacterial host can influence its specificity towards different HSL molecules (50). However, the fact that the C_8-HSL has the strongest response in *E. coli* is an indication that the results obtained here are likely to also occur in *B. cepacia*. It is believed that HSL-protein interaction reduces susceptibility to proteolysis as well as allowing multimerization important for activating transcription (31, 50). It is likely that CepR has a binding pocket to fit molecules with a longer acyl chain. Recently, similar experiments were performed by using a *cep-gfp*-based biosensor showing that CepR from *B. cepacia* H111 responds best to C_8 and other long-chain HSL autoinducers (46). CepR displayed a very low response towards the C_6-HSL, questioning the function of this molecule in *B. cepacia.* However, it cannot be excluded that in the genome of *B. cepacia* there is another *luxR* family gene coding for another LuxR family protein, which displays a better response to the C_6-HSL autoinducer; in this case, this molecule

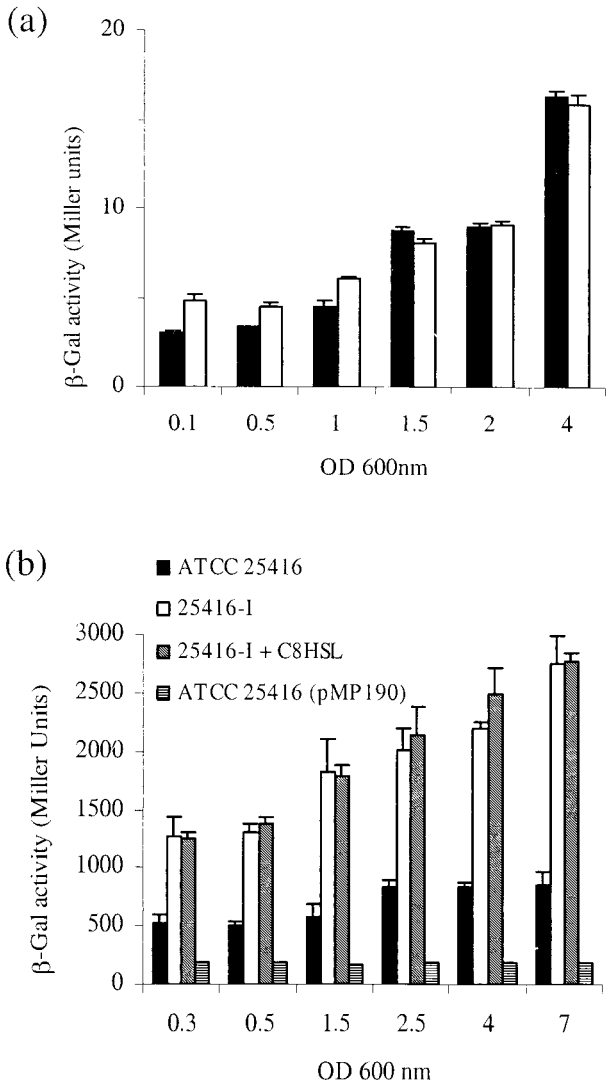

FIG. 4. (a) Production of HSLs at different growth stages from *B. cepacia* ATCC 25416 (filled bars) and from *B. cepacia* ATCC 25416-RPOS (open bars). Values were determined as described in Materials and Methods. (b) *rpoS* promoter activity at different growth stages measured in *B. cepacia* ATCC 25416(pRPR2) (filled bars), *B. cepacia* 25416-I(pRPR2) (open bars), *B. cepacia* 25416-I(pRPR2) plus 100 nM C_8-HSL (shaded bars), and *B. cepacia* ATCC 25416(pMP190) (striped bars). Values were determined as described in Materials and Methods.

might have a biological function. The presence of this molecule could slightly affect our results regarding our estimation in *E. coli*(pSCR1) that a 100 nM concentration of C_8-HSL is regarded as the minimum necessary for the *E. coli*(pSCR1) response (Fig. 1b), since C_6-HSL molecules could interfere with CepR activity.

Various extracellular phenotypes were tested in relation to quorum sensing, and most did not display any alteration in the quorum-sensing-deficient mutant, with the exception of protease activity. Proteolytic activity was significantly lower in the 25416-I mutants; this observation was similar to what was observed in two different *B. cepacia* genomovar III quorum-sensing mutants (23, 29). Interestingly, the two *B. cepacia* genomovar III quorum-sensing mutants displayed either an increase or decrease in siderophore production, whereas in the genomovar I *B. cepacia* strain used in this study there was no alteration of siderophore production. The discrepancies can be attributed to the fact that the strains belong to different genomovars, highlighting the fact that there is no common quorum-sensing phenotype found within the *B. cepacia* complex, with the sole exception of protease activity. Interestingly, polygalacturonase activity was significantly reduced in the quorum-sensing mutants (Table 2), and this was also indirectly observed by the attenuated onion maceration activity (Fig. 2). Polygalacturonase activity in *B. cepacia* ATCC 25416 has been associated with onion maceration, and the plasmid-borne *pehA* gene is a virulence factor in this strain (18). The complemented mutants harboring the *cep* locus in *trans* resulted in higher polygalacturonase activity and higher onion maceration, thus further indicating the involvement of quorum sensing in the onion pathogenicity of this strain. It was not possible, however, to restore wild-type levels of onion maceration activity of the *cepI* and *cepR* mutants by dispersing synthetic C_8-HSL on the onion (data not shown).

The stationary-phase sigma factor RpoS has also been implicated in several gram-negative bacteria in the change in gene expression necessary for the adaptation to stationary phase (17, 38, 47), and it has also been shown to be involved in the regulation of virulence factors (8, 24, 42). We have observed 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 of interest to study whether RpoS and quorum sensing cross-regulate each other since the gene(s) regulated by these two systems are maximally expressed at the stationary phase. It was determined that a genetic background without RpoS has no influence in the accumulation of the HSL molecules produced by *B. cepacia* (Fig. 4a); on the other hand, *rpoS* promoter activity is positively influenced by the absence of the quorum-sensing system (Fig. 4b). This doubling in promoter activity did not reflect an increased resistance to RpoS-regulated stresses; this might be because increasing the promoter activity does not automatically result in an increasing amount of RpoS protein as posttranscriptional regulation of RpoS might occur (22). This increase in *rpoS* promoter activity in the *cepI* mutant 25416-I could not be restored to wild-type levels by the addition to the growth media of synthetic C_8-HSL (Fig. 4b). The reason for this is not known; in addition, it was observed that *rpoS* promoter activity was higher in 25416-I already at low cell densities and activity increased as the cells entered the stationary phase (Fig. 4b). 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 the stationary phase (23). It is similar, however, to what happens with *E. coli* since RpoS levels are mainly controlled at the posttranscriptional and posttranslational levels (22). In *R. solanacearum* it has been reported that RpoS regulates the HSL production by the decrease in *solR* and *solI* expression (13). Similarly, in *P. aeruginosa*, quorum sensing and *rpoS* cross-regulate each other (28, 51). Future work will determine how quorum sensing regulates *rpoS* and whether the two systems

are involved in the regulation of similar phenotypes of *B. cepacia*.

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