The *cep* quorum-sensing system of *Burkholderia cepacia* H111 controls biofilm formation and swarming motility

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***Burkholderia cepacia* and *Pseudomonas aeruginosa* often co-exist as mixed biofilms in the lungs of patients suffering from cystic fibrosis (CF). Here, the isolation of random mini-Tn5 insertion mutants of *B. cepacia* H111 defective in biofilm formation on an abiotic surface is reported. It is demonstrated that one of these mutants no longer produces *N*-acylhomoserine lactones (AHLs) due to an inactivation of the *cepR* gene. *cepR* and the *cepI* AHL synthase gene together constitute the *cep* quorum-sensing system of *B. cepacia*. By using a gene replacement method, two defined mutants, H111-I and H111-R, were constructed in which *cepI* and *cepR*, respectively, had been inactivated. These mutants were used to demonstrate that biofilm formation by *B. cepacia* H111 requires a functional *cep* quorum-sensing system. A detailed quantitative analysis of the biofilm structures formed by wild-type and mutant strains suggested that the quorum-sensing system is not involved in the regulation of initial cell attachment, but rather controls the maturation of the biofilm. Furthermore, it is shown that *B. cepacia* is capable of swarming motility, a form of surface translocation utilized by various bacteria to rapidly colonize appropriate substrata. Evidence is provided that swarming motility of *B. cepacia* is quorum-sensing-regulated, possibly through the control of biosurfactant production. Complementation of the *cepR* mutant H111-R with different biosurfactants restored swarming motility while biofilm formation was not significantly increased. This result suggests that swarming motility *per se* is not essential for biofilm formation on abiotic surfaces.**

Keywords: cystic fibrosis, *N*-acylhomoserine lactone, biosurfactant

INTRODUCTION

To date, most research in molecular microbiology has focused on the analysis of planktonic cells in pure cultures and it was not until recently that it was fully appreciated that in nature bacteria predominantly exist as sessile, surface-associated communities, referred to as biofilms (for recent reviews see O'Toole *et al.*, 2000; Tolker-Nielsen & Molin, 2000; Watnick & Kolter, 2000). Many persistent and chronic bacterial infections, including periodontitis, otitis media, biliary tract infection and endocarditis, are intrinsically linked to the

formation of biofilms. Moreover, various medical implants are prone to colonization by pathogenic bacteria (Costerton *et al.*, 1999). Bacterial biofilm infections are particularly problematic as sessile bacteria can withstand host immune responses and are markedly more resistant to antibiotics (up to 1000-fold) and biocides than cells grown in suspension (Schierholz *et al.*, 1999; Xu *et al.*, 2000).

The opportunistic pathogenic bacterium *Pseudomonas aeruginosa* is capable of chronically colonizing the lungs of patients suffering from cystic fibrosis (CF), the most common lethal inherited disease among the Caucasian population (Koch & Høiby, 1993; Govan & Deretic, 1996; Tümmler & Kiewitz, 1999). During chronic infection, *P. aeruginosa* produces copious amounts of alginate, which forms a matrix completely embedding

Abbreviations: AHL, *N*-acylhomoserine lactone; CF, cystic fibrosis; CLSM, confocal laser scanning microscopy; GFP, green fluorescent protein; C8-HSL, *N*-octanoylhomoserine lactone; C6-HSL, *N*-hexanoylhomoserine lactone; 3-oxo-C6-HSL, *N*-(3-oxohexanoyl)homoserine lactone.

the cells, and becomes highly resistant to antibiotic treatment. These observations led to the suggestion that *P. aeruginosa* may exist as a biofilm in the CF lung (Lam *et al.*, 1980; Costerton *et al.*, 1999). This hypothesis was recently corroborated through profiling of *N*-acylhomoserine lactone (AHL) signal molecules (Singh *et al.*, 2000). *Burkholderia cepacia* has been recognized as another important pathogen in patients with CF. Infection with *B. cepacia* often occurs in patients who are already colonized with *P. aeruginosa*. In fact, it has been suggested that *P. aeruginosa* exoproducts may modify the epithelial cell surface of the lung such that attachment of *B. cepacia* is facilitated (Saiman *et al.*, 1990). Co-colonization can result in three clinical outcomes: asymptomatic carriage, slow and continuous decline in lung function, or, for approximately 20% of the patients, fulminant and fatal pneumonia, the so-called 'cepacia syndrome' (Isles *et al.*, 1984).

In both *P. aeruginosa* (for reviews see de Kievit & Iglewski, 2000; Parsek & Greenberg, 2000; Williams *et al.*, 2000) and *B. cepacia* (Lewenza *et al.*, 1999), expression of various virulence factors is controlled by AHL-dependent quorum-sensing systems. These regulatory systems ensure that pathogenic traits are only expressed when the bacterial population density is high enough to overwhelm the host before it is able to mount an efficient response. Interestingly, for *P. aeruginosa* it has been demonstrated that the architecture of biofilms formed on an abiotic surface is also quorum-sensing-controlled (Davies *et al.*, 1998). These results argue in favour of functional overlaps between factors necessary for biofilm formation and pathogenicity. The quorum-sensing system of *B. cepacia* K56-2 (genomovar III) has been recently identified (Lewenza *et al.*, 1999). This density-dependent regulatory system relies on two proteins: the AHL synthase CepI, which directs the synthesis of *N*-octanoylhomoserine lactone (C8-HSL) and, as a minor product, *N*-hexanoylhomoserine lactone (C6-HSL) (Gotschlich *et al.*, 2001), and CepR, which after binding of C8-HSL is thought to activate or repress transcription of target genes. The *cep* system was demonstrated to positively regulate protease production and to repress synthesis of the siderophore ornibactin (Lewenza *et al.*, 1999). Since the two bacteria not only form mixed biofilms in CF lungs but also utilize the same chemical language, it appears likely that the two species synergistically enhance the others' virulence (McKenney *et al.*, 1995).

The recent development of a simple biofilm assay has greatly facilitated the analysis of the genetic mechanisms underlying biofilm formation. In this assay, bacteria are grown in the wells of microtitre dishes in which the cells attach to the abiotic surface. Following removal of planktonic cells, the established biofilm is quantified after staining with crystal violet. Over the past few years, this assay has been extensively used to identify genes involved in biofilm formation in a number of bacteria, including *Escherichia coli* (Pratt & Kolter, 1998), *Pseudomonas fluorescens* (O'Toole & Kolter, 1998a), *P. aeruginosa* (O'Toole & Kolter, 1998b), *Vibrio*

cholerae (Watnick & Kolter, 1999) and *Streptococcus gordonii* (Loo *et al.*, 2000). In the present study we have employed this assay to isolate random transposon insertion mutants in *B. cepacia* H111 that are defective in biofilm formation on a polystyrene surface. One of these mutants is demonstrated to bear the transposon within the *cepR* gene. This finding prompted us to investigate the role of the *cep* quorum-sensing system in the strain's ability to form biofilms. It is shown that both biofilm formation and swarming motility are *cep*-regulated phenotypes.

METHODS

Strains, plasmids and growth conditions. Strains and plasmids used in this study are listed in Table 1. Unless otherwise stated, the strains were grown at 37 °C in modified Luria-Bertani (LB) broth (Andersen *et al.*, 1998) or AB minimal medium (Clark & Maaløe, 1967) supplemented with 10 mM citrate. Solid media were routinely solidified with 1.5% (w/v) agar. Growth media for examination of swimming and swarming motility contained 0.3% and 0.4% agar, respectively (Eberl *et al.*, 1996). Antibiotics were added as required at final concentrations of 100 µg ampicillin ml⁻¹, 10 µg tetracycline ml⁻¹, 20 µg gentamicin ml⁻¹ and 10 µg chloramphenicol ml⁻¹. Kanamycin was used at 50 µg ml⁻¹ for *E. coli* and 100 µg ml⁻¹ for *B. cepacia*. Tellurite concentration was 100 µg ml⁻¹. For complementation of H111-I, 200 nM C8-HSL and/or 200 nM C6-HSL were added. Growth of liquid cultures was monitored spectrophotometrically by an Ultrospec Plus spectrophotometer (Pharmacia) by measurement of optical density at 600 nm.

Conjugative plasmid transfer. Plasmids were delivered to *B. cepacia* by triparental mating as described by de Lorenzo & Timmis (1994). Briefly, donor and recipient strains and also the helper strain *E. coli* HB101(pRK600) were grown at 37 °C overnight in 5 ml LB supplied with the appropriate antibiotics. Following subculturing to an OD₆₀₀ of 0.9, the cells from 2 ml of culture were harvested, washed and resuspended in 200 µl LB. Donor and helper cells (100 µl each) were mixed and incubated for 30 min at room temperature. Recipient cells (200 µl) were added and the mixture was spot-inoculated onto the surface of prewarmed LB agar plates. After overnight incubation at 37 °C, the cells were scraped off and were resuspended in 1 ml 0.9% NaCl. Serial dilutions were plated on LB medium containing antibiotics for counter-selection of donor, helper and untransformed recipient cells.

DNA manipulations and nucleotide sequencing. Cloning, restriction enzyme analysis and transformation of *E. coli* were performed essentially as described by Sambrook *et al.* (1989). PCR was performed using the TaKaRa rTaq DNA polymerase (TaKaRa Shuzo). Plasmid DNA was isolated with the QIAprep Spin Miniprep kit and chromosomal DNA from *B. cepacia* was purified with the DNeasy Tissue kit. DNA fragments were purified from agarose gels using the QIAquick Gel Extraction kit (all kits were from Qiagen).

For complementation of *B. cepacia* H111-R, we constructed plasmid pBAH27 (*cepR*⁺) as follows. The *cepR* gene was PCR-amplified using primers *cepR*-R (5'-GGGGTACCAACCTG-ACAAGTATGACAGCG-3') and *cepR*-OV (5'-GGGGTACCGGATGAGCATGGAGAAAAGC-3') (*KpnI* restriction sites are underlined). Following digestion with *KpnI*, the PCR fragments were inserted into the broad-host-range vector pBBR1MCS-5 cut with the same enzyme. The plasmid

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
<i>E. coli</i>		
XL1-Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacI^q</i> ZΔM15 Tn10 (Tet ^R)]	Stratagene
MT102	<i>araD139 (ara-leu)7697 Δlac thi hsdR</i>	Laboratory collection
CC118 (λpir)	Δ(<i>ara-leu</i>) <i>araD</i> Δ <i>lacX74 galE galK phoA thi-1 rpsE rpoB argE</i> (Am) <i>recA</i> , λpir lysogen	Herrero <i>et al.</i> (1990)
<i>B. cepacia</i>		
H111	CF isolate from a patient at the Medizinische Hochschule Hannover (1993), genomovar III	Römling <i>et al.</i> (1994); Gotschlich <i>et al.</i> (2001)
m64	CepR::Tn5Km2- <i>luxCDABE</i> derivative of H111	This study
H111-I	Km ^r , <i>cepI</i> ::Km mutant of H111	This study
H111-R	Km ^r , <i>cepR</i> ::Km mutant of H111	This study
Plasmids		
pAG	Gm ^r Km ^r , pEX18Gm derivative for inactivation of <i>cepI</i>	This study
pBAH27	pBBR1MCS-5 containing the <i>cepR</i> gene of <i>B. cepacia</i> H111	This study
pBAH33	Gm ^r Km ^r , pEX19Gm derivative for inactivation of <i>cepR</i>	This study
pBBR1MCS-5	Gm ^r , broad-host-range cloning vector	Kovach <i>et al.</i> (1995)
pEX18Gm	Gm ^r , <i>oriT⁺ sacB⁺</i> , gene replacement vector with multiple cloning site from pUC18	Hoang <i>et al.</i> (1998)
pEX19Gm	Gm ^r , <i>oriT⁺ sacB⁺</i> , gene replacement vector with multiple cloning site from pUC19	Hoang <i>et al.</i> (1998)
pGEM-3Zf(+)	Ap ^r , multicopy cloning vector	Promega
pMH94	Ap ^r Km ^r , delivery vector for mini-Tn5 Tel ^r <i>kilAtelAB-P_{AI/04/03}-gfp mut3-T₀-T₁</i>	M. Hentzer & M. R. Parsek (unpublished)
pRK600	Cm ^r , ColE1oriV, RK-2Mob ⁺ RK2-Tra ⁺ , helper plasmid in triparental matings	de Lorenzo & Timmis (1994)
pSB403	Tc ^r , <i>luxRI</i> :: <i>luxCDABE</i> transcriptional fusion, bioluminescent AHL sensor plasmid	Winson <i>et al.</i> (1998b)
pUTmini-Tn5Km2- <i>luxCDABE</i>	Km ^r , delivery vector for miniTn5Km2- <i>luxCDABE</i>	Winson <i>et al.</i> (1998a)

containing the insert in the orientation placing the *cepR* gene downstream of the P_{lac} promoter of the cloning vector was chosen and this construct was designated pBAH27. For flow-chamber experiments, the strains were tagged with green fluorescent protein (GFP). This was accomplished by the insertion of a P_{AI/04/03}-*gfp*-T0-T1 transposon cassette (Andersen *et al.*, 1998) into the chromosomes of target strains using the suicide construct pMH94 (M. Hentzer & M. R. Parsek, unpublished results). Plasmid pMH94 was delivered to target strains by conjugative transfer and integrants were selected on PIA medium (Becton Dickinson Biosciences) containing tellurite.

Sequencing was performed by the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977) in a LI-COR 4200 DNA sequencer. The primer 5'-CAGATCTGATCAAGAG-ACAG-3', which binds to the I-end of the Tn5 transposon, was used for determination of the transposon insertion point in *B. cepacia* m64. DNA sequences were compared to other sequences in GenBank using the on-line BLAST search engine at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>).

Construction of a *B. cepacia* H111 mutant bank. The hybrid transposon mini-Tn5Km2-*luxCDABE* was randomly inserted into the chromosome of *B. cepacia* H111 by the triparental mating procedure described above. Transconjugants were selected on LB medium containing kanamycin and tetracycline. These random insertion mutants were picked and grown in 150 µl LB medium in the wells of polypropylene MicroWell dishes (Nunc). For storage, 75 µl 50% (v/v) glycerol was added and the dishes were frozen at -80 °C.

Screen for mutants defective in biofilm formation. Biofilm formation in polystyrene microtitre dishes was assayed essentially as described by O'Toole & Kolter (1998a) and Pratt & Kolter (1998) with a few modifications. Cells were grown in the wells of the microtitre dishes in 100 µl AB medium supplemented with 10 mM citrate for 48 h at 30 °C. The medium was then removed and 100 µl of a 1% (w/v) aqueous solution of crystal violet was added. Following staining at room temperature for 20 min, the dye was removed and the wells were washed thoroughly. For quantification of attached cells, the crystal violet was solubilized in a 80:20

(v/v) mixture of ethanol and acetone and the absorbance was determined at 570 nm.

Detection and characterization of AHLs. Production of AHLs was investigated with the aid of the bioluminescent plasmid sensor pSB403 (Winson *et al.*, 1998b). This sensor plasmid contains the *Photobacterium fischeri luxR luxI* gene together with the *luxI* promoter region as a transcriptional fusion to the bioluminescence genes *luxCDABE* of *Photobacterium luminescens*. The quorum-sensing system of *Photobacterium fischeri* relies on N-(3-oxohexanoyl)homoserine lactone (3-oxo-C6-HSL) and the sensor plasmid consequently exhibits the highest sensitivity for this AHL molecule. However, several other AHL molecules are detected by the sensor, albeit with somewhat reduced sensitivity (Winson *et al.*, 1998b; Geisenberger *et al.*, 2000). Bioluminescence was detected either with the highly sensitive photon-counting camera C2400-40 (Hamamatsu Photonics) or by exposure to an X-ray film. For more detailed analysis, the AHL molecules were extracted from spent culture supernatants of the strains, separated by TLC and AHL spots were visualized by overlaying the TLC plates with soft agar seeded with the sensor strain *E. coli* MT102(pSB403) as described previously (Shaw *et al.*, 1997; Geisenberger *et al.*, 2000). Routinely, AHLs were extracted twice with dichloromethane (250:100 supernatant/dichloromethane) from 250 ml sterile-filtered supernatants of *B. cepacia* cultures grown in AB minimal medium containing 10 mM citrate at 30 °C to an OD₆₀₀ of 1.0. The combined extracts were dried over anhydrous magnesium sulfate, filtered and evaporated to dryness. Residues were dissolved in 250 µl ethyl acetate. Samples (10 µl) were then applied to C₁₈ reversed-phase TLC plates (Merck no. 1.15389) and dried with a stream of cold air. Samples were separated by using methanol in water (60%, v/v) as the mobile phase. For detection of AHLs, the TLC plate was overlaid with a thin film of LB agar (143 ml) seeded with 7 ml of an exponentially grown AHL biosensor and was then incubated at 30 °C for 24 h. The tentative identification of AHLs present in spent culture supernatant extracts was achieved by comparison of mobilities (R_f values) relative to those for the synthetic AHL standards.

For quantification of AHL signal molecules, 100 µl of filter-

Fig. 1. Physical and genetic map of the *cep* quorum-sensing region of *B. cepacia* H111. Genes are marked by open arrows, with the direction of the arrowheads indicating the direction of transcription. A palindrome sequence in the *cepI* promoter region that is highly homologous to the *lux* box consensus sequence is indicated. Relevant restriction sites are shown. The location of the mini-Tn5Km2-*luxCDABE* element in *B. cepacia* m64 is indicated by a triangle. The positions of the *npt* cassettes in the *cepI* mutant H111-I and the *cepR* mutant H111-R are indicated by circles.

sterilized supernatants of cultures grown in LB medium to an OD_{600} of 3.0 was added to 100 μ l of an exponential culture of *E. coli* MT102(pSB403) in the wells of a FluoroNunc Polysorp microtitre dish. Following incubation at 30 °C for 6 h, bioluminescence was measured with a Lamda Fluoro 320 Plus reader (Bio-Tek Instruments).

Exoenzyme and siderophore production. Exoenzyme and siderophore production was tested by streaking strains on appropriate indicator plates. Proteolytic activity was determined on LB medium supplemented with 2% skim milk, chitinolytic activity on ethylene glycol chitin agar (Connell *et al.*, 1998) and lipolytic activity on tributyrin agar base containing 1% glycerol tributyrate (both Merck). Clear haloes around the colonies after incubation at 37 °C overnight indicated exoenzyme activity. Siderophore production was tested by growing strains on CAS agar (Schwyn & Neilands, 1987) for 24 h. Siderophores remove the iron from the CAS dye complex, resulting in a colour change around the colonies from blue to orange.

Construction of *B. cepacia* H111 *cepI* and *cepR* mutant strains. Defined *cep* mutants (Fig. 1) were constructed by the gene replacement method described by Hoang *et al.* (1998). For the construction of a *cepR* mutant, two DNA fragments were PCR-amplified: an 850 bp *EcoRI*–*SacI* fragment spanning the intergeneric region plus the first 75 bp of *cepR* using the primer pair intercep-f-*Eco* (5'-GGAATTCGAGATCCG-CCGCGAGTTCG-3') and intercep-r-*Sac* (5'-GATCCGCTG-GAAGAGCTCC-3'), and a 760 bp *SphI*–*HindIII* fragment containing the 3' region of the *cepR* gene using the primer pair cepR-f-*Sph* (5'-ACATGCATGCGCTCGGATTCGAATAC-TGC-3') and cepR-r-*Hind* (5'-CCCAAGCTTAGAAGCTC-GAGCAGATCGC-3'). Using the restriction sites introduced by the PCR primers (respective sites are underlined), these two DNA fragments were successively inserted into the compatible sites of the gene replacement vector pEX19Gm (Hoang *et al.*, 1998). Next, the *npt* gene from transposon Tn903 (Oka *et al.*, 1981), which confers resistance to kanamycin, was cloned as a 1.7 kb *Bam*HI fragment into the vector cut with the same enzyme. The final construct, which was designated pBAH33, was transferred to *B. cepacia* H111 and integrants were selected on LB medium containing kanamycin and tetracycline. To screen for gene replacement mutants, Kan^r clones were tested for gentamicin sensitivity as the gentamicin resistance gene is lost in the case of a double crossover event. One mutant, which was designated *B. cepacia* H111-R, was chosen and the correct genetic structure of the strain was confirmed by Southern Blot analysis. Construction of a defined *cepI* mutant was performed as described for the *cepR* mutant, except that the gene replacement vector pEX18Gm and two

different PCR fragments were used. An 800 bp *EcoRI*–*SacI* fragment spanning the intergeneric region plus 100 bp of *cepR* was amplified with the primer pair igR-f-*Eco* (5'-GGAATTC-CCAGTATTCGAATCCGAGCCGC-3') and igR-r-*Sac* (5'-CGAGCTCGGGATGTCCTCGGATCTGTGC-3'), and a 650 bp *Bam*HI–*HindIII* fragment containing the 5' region of *cepI* was amplified using the primer pair cepI-f-*Bam* (5'-CGGGATCCCGCCTTCGTTACGAGGAAGGG-3') and cepI-r-*Hind* (5'-CCCAAGCTTGGGCGCGCTTCGGC-TCAGG-3'). The final gene replacement construct was designated pAG and the respective *B. cepacia* H111 *cepI* mutant was named H111-I.

Monitoring of biofilm formation by confocal laser scanning microscopy (CLSM) and image analysis. Biofilms were grown in artificial flow cells supplied with AB medium and containing 1 mM glucose (ABG). The flow system was assembled and prepared as described previously (Christensen *et al.*, 1999). The substratum consisted of a microscope glass coverslip (Knittel Gläser). Overnight cultures in ABG medium were subcultured to an OD_{600} of 0.7 before dilution in 0.9% NaCl to an OD_{600} of 0.1. Aliquots (300 μ l) of these dilutions were used to inoculate the flow channels. Medium flow was kept at a constant rate of 0.7 mm s⁻¹ by a Watson-Marlow 205S peristaltic pump. Incubation temperature was 30 °C.

Microscopic inspection and image acquisition were performed on a confocal laser scanning microscope (TCS4D; Leica Lasertechnik) equipped with a 63 $\times$ /1.32–0.6 oil objective. For statistical evaluation of biofilm structures, a 40 $\times$ /0.75 air objective was used. Image scanning was carried out with the 488 nm laser line of an Ar/Kr laser. Captured images were visualized using the IMARIS software package (Bitplane) running on a Silicon Graphics Indigo 2 workstation.

For statistical evaluation of biofilm structures, three independent rounds of biofilm experiments were performed, and in each round, each strain was grown in two separate channels. Seven image stacks were taken of each channel every 24 h for 7 d after inoculation. These images were analysed by the computer program COMSTAT, which comprises various features for quantifying three-dimensional biofilm image stacks (Heydorn *et al.*, 2000). The parameters used for characterization of biofilm architecture included biomass, substratum coverage, mean thickness and roughness coefficient.

RESULTS

Screening for biofilm-defective mutants

To determine the optimum experimental conditions for attachment of *B. cepacia* H111 to abiotic surfaces, biofilm assays were carried out under various conditions. While the strain formed thick biofilms in polystyrene microtitre dishes, no attachment was observed in dishes made of polypropylene. In contrast to other bacteria (O'Toole & Kolter, 1998a; Danese *et al.*, 2000; Loo *et al.*, 2000), biofilm formation by *B. cepacia* H111 is virtually independent of medium composition. AB minimal medium supplemented with glucose or citrate supported surface colonization as well as rich media such as LB. Furthermore, incubation temperatures ranging from 30 to 37 °C did not significantly affect biofilm yields (data not shown). For routine biofilm assays we used AB minimal medium containing 10 mM citrate and incubated the microtitre dishes for 48 h at 30 °C.

Fig. 2. AHL production by *B. cepacia* H111 is subject to positive feedback regulation. Aliquots (100 µl) of filter-sterilized culture supernatants of wild-type H111, the *cepI* mutant H111-I, the *cepR* mutants m64 and H111-R and the complemented *cepR* mutant H111-R(pBAH27) were mixed with an exponential culture of the AHL sensor MT102(pSB403) in a microtitre dish. Following incubation for 6 h, bioluminescence was quantified with a luminometer. Error bars represent the standard deviation of the mean for four independent wells. Bioluminescence was also visualized with the aid of a photon camera (pictures shown below the x axis). C8-HSL (10 µM) and C6-HSL (1 µM) standards were included as controls.

Table 2. Phenotypic characterization of *B. cepacia* H111 and *cep*-defective mutants

Production of AHLs was determined by testing culture supernatants for stimulation of the bioluminescent sensor plasmid pSB403. Synthesis of extracellular hydrolytic enzymes and siderophore activities were assessed by streaking strains on appropriate indicator plates as described in Methods. Swimming and swarming motility was tested on media solidified with 0.3% and 0.4% agar, respectively. Wild-type H111, the *cepR* mutant H111-R, the complemented *cepR* mutant H111-R(pBAH27) and the *cepI* mutant H111-I in the presence or absence of C8-HSL (200 nM) were investigated. +, Activity exhibited by the wild-type; (+) significantly reduced activity; –, no detectable activity; NA, not applicable.

Phenotype	H111	H111-R	H111-R (pBAH27)	H111-I	H111-I + AHL
AHL	+	–	+	–	NA
Protease	+	–	+	–	+
Chitinase	+	(+)	+	(+)	+
Lipase	+	+	+	+	+
Siderophore	+	(+)	+	(+)	+
Swarming	+	–	+	–	+
Swimming	+	+	+	+	+

The suicide vector pUT (de Lorenzo & Timmis, 1994) was used to deliver the hybrid transposon mini-Tn5Km2-*luxCDABE* (Winson *et al.*, 1998a) into the *B. cepacia* H111 chromosome. A collection of 5000 random insertion mutants was screened for ability to form biofilms. A total of eighteen mutants which were to different degrees defective in biofilm formation was obtained (data not shown). During the course of a detailed phenotypical characterization of these mutants we noticed that one, m64, was deficient in the production of AHL signal molecules. As shown in Fig. 2, the wild-type strain H111 strongly activated the bioluminescent AHL sensor plasmid pSB403 while no activation was observed with m64. This result is reminiscent of the situation found with *P. aeruginosa*, for which it has been

shown that development of a mature biofilm is quorum-sensing-regulated (Davies *et al.*, 1998). We therefore focused our further investigations on the analysis of this mutant.

Since *B. cepacia* H111 produces two AHL molecules, C8-HSL and C6-HSL, in a ratio of 10:1 (Gotschlich *et al.*, 2001), we next extracted the signal molecules from spent culture supernatants of the wild-type and the mutant and analysed them by TLC. This analysis showed that the mutant is impaired in the production of both molecules (data not shown). Addition of 200 nM C8-HSL to the growth medium did not restore biofilm formation (data not shown). To further characterize the nature of the transposon insertion in strain m64, the mutated locus was cloned into the cloning vector pGEM-3Zf(+) as a *SphI* fragment, selecting for transposon-encoded kanamycin resistance. The resulting plasmid contains part of the transposon and approximately 3 kb of chromosomal DNA upstream of the transposon insertion point. Nucleotide sequence analysis of the flanking DNA revealed that the transposon had inserted into the *cepR* gene of *B. cepacia* H111 at position 356 (Fig. 1; GenBank accession no. AF330020). These data are fully consistent with previous results that showed that expression of *cepI* and thus AHL signal production is regulated by CepR (Lewenza *et al.*, 1999). More importantly, these results suggest that the *cep* quorum-sensing system is involved in the regulation of biofilm formation in *B. cepacia* H111.

Construction and characterization of defined *B. cepacia* H111 *cep* mutants

To investigate the role of the *cepIR* genes in biofilm formation in greater detail, we constructed site-directed insertion mutations in the two genes by using a gene replacement method (see Methods for details and Fig. 1). The genetic structure of the two mutants, which were designated H111-I and H111-R, respectively, was confirmed by Southern blot analysis (data not shown).

As expected, neither the *cepI* mutant H111-I nor the *cepR* mutant H111-R produced detectable amounts of

Fig. 3. The *cep* quorum-sensing system controls biofilm formation by *B. cepacia* H111. Strains were grown in AB minimal medium supplemented with 10 mM citrate in the wells of polypropylene microtitre dishes. After incubation for 48 h at 30 °C, planktonic cells were removed and attached cells were stained with crystal violet. Biofilms formed by wild-type H111, the *cepR* mutants m64 and H111-R, the complemented *cepR* mutant H111-R(pBAH27) and the *cepI* mutant H111-I in the presence or absence of C8-HSL (200 nM) are shown in (a); quantification of the biofilm-associated dye is shown in (b). Error bars represent the standard deviation of the mean for six independent wells.

AHLs (Fig. 2). However, production of AHLs was restored to wild-type levels when H111-R was complemented with plasmid pBAH27, which contains the *cepR* gene inserted into the broad-host-range vector pBBR-1MCS-5.

B. cepacia produces different siderophores and a number of exoenzymes that are thought to be pathogenesis factors in humans as well as in plants (Lonon *et al.*, 1988; McKevitt *et al.*, 1989; Gessner & Mortensen, 1990; Yohalem & Lorbeer, 1994; Darling *et al.*, 1998). In a recent study it was shown that the *cep* system of *B. cepacia* K56-2 is involved in the regulation of the synthesis of extracellular enzymes and siderophores (Lewenza *et al.*, 1999). We therefore tested the *B. cepacia* H111 wild-type and the two mutants H111-I and H111-R for the production of extracellular protease, lipase, chitinase and siderophores on appropriate indicator plates. The results of these investigations are summarized in Table 2. Consistent with the results reported by Lewenza *et al.* (1999), both mutants showed a clear reduction in protease activity. Furthermore, proteolytic activities of the mutants were completely restored when mutant H111-I was grown in the presence of 200 nM C8-HSL or when plasmid pBAH27 (*cepR*⁺) was transferred to mutant H111-R. Both mutants were found to produce significantly lowered amounts of siderophores as assessed on CAS indicator plates. As for proteolytic activity, these defects were restored to wild-type levels by the external addition of 200 nM C8-HSL to H111-I or by complementation of H111-R with plasmid pBAH27 (*cepR*⁺). These data are in contrast to the results of the above-mentioned study, which showed that inactivation of either *cepI* or *cepR* results in an up-regulation of siderophore production in *B. cepacia* K56-2. Most likely, this apparent discrepancy can be attributed to the different strains used in the studies. Likewise, while Lewenza *et al.* (1999) observed reduced lipase activity

with the *cepR* but not with the *cepI* mutant of K56-2, we were unable to detect any difference in the lipase activities of H111, H111-I and H111-R. Chitinase activity was slightly reduced in the two mutants when compared with the wild-type, and since complementation (as described above) restored the defects we suggest that chitinase production in *B. cepacia* H111 is, at least in part, regulated by quorum sensing.

The *cep* system of *B. cepacia* H111 controls biofilm maturation

We next tested the two mutants H111-I and H111-R for their abilities to form biofilms in microtitre dishes as described above. In agreement with our initial observation with strain m64, we found that both mutants were defective in biofilm formation (Fig. 3). However, addition of 200 nM C8-HSL to the medium completely restored biofilm formation by mutant H111-I. Likewise, H111-R harbouring plasmid pBAH27 formed wild-type biofilms. As one reason for lowered biofilm formation capability is reduced growth, we determined the growth rates of H111, H111-I and H111-R in AB minimal medium supplemented with 10 mM citrate. Wild-type and mutant strains were found to grow equally well in liquid culture (data not shown).

To further analyse the role of the *cep* system in biofilm formation, we employed artificial flow cells, which allow biofilm development to be followed on a glass surface under highly defined conditions in real time (Christensen *et al.*, 1999). Moreover, by the use of a confocal laser scanning microscope the three-dimensional structure of the biofilm can be reconstructed. For this analysis, it was necessary to tag *B. cepacia* H111 and the two mutants H111-I and H111-R with GFP. This was accomplished by inserting a P_{A1/04/03}-*gfp*-T0-T1 transposon cassette (Andersen *et al.*, 1998) randomly

Fig. 4. Biofilm formation by *B. cepacia* H111 is quorum-sensing-regulated. Flow chambers were inoculated with *gfp*-tagged derivatives of wild-type H111 and of the *cepI* mutant H111-I in the presence or absence of 200 nM C8-HSL. The CLSM pictures shown were taken 4 d after inoculation. The larger central plots are simulated fluorescence projections. Shown in the right and lower frames are vertical sections through the biofilms. Bars, 20 μ m.

into the chromosome of each of the three strains using the suicide plasmid pJMT6 (Sanchez-Romero *et al.*, 1998). The tagged strains used for further investigations were carefully tested with respect to growth rates in liquid medium and biofilm formation in microtitre dishes and were found to be indistinguishable from the parental strains (data not shown).

Parallel flow chambers were inoculated with each of the three GFP-tagged strains and biofilm development was monitored on a daily basis for 7 d. Visual inspection of CLSM images revealed that the biofilms formed by the two mutants not only differed in their substratum coverage and thickness, as had been anticipated from the microtitre plate assays, but also exhibited strikingly different structures (Fig. 4 and data not shown). Both wild-type and mutant strains formed characteristic microcolonies after initial surface attachment. However, while wild-type biofilms rapidly matured and covered most of the available surface space within 24 h, mutant biofilms were arrested in the microcolony stage and never colonized the entire surface during the course of the experiment.

To more accurately describe the differences in the biofilms formed by the wild-type and the *cep* mutants, we employed the computer program COMSTAT, which was recently developed for the quantitative characterization of biofilm structures (Heydorn *et al.*, 2000). Out of ten image analysis features which the program provides for quantifying three-dimensional image stacks acquired by CLSM, we chose the following: biomass, mean thickness, substratum coverage and roughness coefficient. To generate data of statistical value, three independent rounds of biofilm experiments were performed. In each round, two flow chamber channels were inoculated with each strain tested. Seven image stacks per channel were taken every 24 h for 7 days and these were analysed with COMSTAT (Heydorn *et al.*, 2000). In

full agreement with the visual impression, the coefficients for mean thickness and for biomass were greatly reduced for the *cep* mutants when compared with the wild-type (Fig. 5). The two mutants colonize the surface less efficiently than the wild-type, a fact that is reflected by a higher value for substratum coverage for the latter strain. The roughness coefficient is a measure of the variance of biofilm thickness and the higher values of this coefficient for mutant biofilms indicate that they are more heterogeneous than wild-type biofilms. Importantly, in the presence of 200 nM C8-HSL, strain H111-I forms a biofilm that is completely indistinguishable from the one of the wild-type strain (Fig. 4 and Fig. 5a). Likewise, strain H111-R harbouring plasmid pBAH27 (*cepR*⁺) forms a biofilm with a typical wild-type structure (Fig. 5b).

In conclusion, these results clearly show that the *cep* quorum-sensing system of *B. cepacia* H111 is involved in the control of biofilm formation. More specifically, our data suggest that the *cep* system may not be important for the initial attachment of cells to the surface but is essential for the differentiation of microcolonies, a process that is required for the development of a mature biofilm.

Swarming motility of *B. cepacia* H111 is regulated by the *cep* system

Genetic studies have shown that the formation of a mature biofilm proceeds through an ordered series of steps (for recent reviews see Pratt & Kolter, 1999; Watnick & Kolter, 2000; O'Toole *et al.*, 2000). In this model, motility plays a major role in biofilm formation. Flagella-mediated motility is believed to be required to overcome repulsive forces at the surface of the substratum. Furthermore, once the initial contact to the surface is established, cells are thought to move on top

Fig. 5. Quantification of biofilm structures. Biofilms of wild-type H111 (●), and (a) the *cepI* mutant H111-I in the presence (▲) or absence (■) of 200 nM C8-HSL, and (b) the *cepR* mutant H111-R (▼) and the complemented *cepR* mutant H111-R (pBAH27) (◆) were grown in artificial flow cells. CLSM pictures were taken daily for 1 week and these pictures were analysed by the computer program COMSTAT (Heydorn *et al.*, 2000). Parameters calculated were biomass, mean biofilm thickness, substratum coverage and roughness coefficient. Mean values for seven independent CLSM pictures are shown with standard deviations.

Fig. 6. Swarming motility of *B. cepacia* H111 is quorum-sensing-regulated. Swarming of wild-type H111, the *cepR* mutant H111-R in the presence or absence of the biosurfactant serrawettin W2 ($1 \mu\text{g ml}^{-1}$) and the complemented *cepR* mutant H111-R(pBAH27) was tested on minimal medium solidified with 0.4% agar and supplemented with 0.1% Casamino acids. All strains exhibited limited swimming motility at the point of inoculation under the assay conditions used.

of the substratum to form microcolonies. Finally, these microcolonies undergo a differentiation process which leads to the development of a typical three-dimensional biofilm architecture.

For *P. aeruginosa*, it has been shown that aggregation of the cells to microcolonies is dependent on twitching motility, a special form of surface translocation that depends on type IV pili (O'Toole & Kolter, 1998b). For *V. cholerae* El Tor and *E. coli*, it has been suggested that flagella-driven motility is not only important for initial attachment of cells to the substratum but also for translocation along the surface in a process that leads to the formation of microcolonies (Pratt & Kolter, 1998; Watnick & Kolter, 1999). The importance of motility for biofilm formation, together with the fact that different forms of bacterial motility, including swimming of *Yersinia pseudotuberculosis* (Atkinson *et al.*, 1999), twitching of *P. aeruginosa* (Glessner *et al.*, 1999) and swarming of *Serratia liquefaciens* (Eberl *et al.*, 1996) and *P. aeruginosa* (Köhler *et al.*, 2000) are quorum-sensing-regulated, prompted us to investigate whether the *cep* system of *B. cepacia* H111 is involved in the control of motility.

When cells of *B. cepacia* H111 are point-inoculated into AB minimal medium which is supplemented with 10 mM citrate and solidified with 0.3% agar, they swim through the water channels in the agar giving rise to typical chemotactic rings. Swimming behaviour of the

cep mutants and the wild-type was completely indistinguishable, indicating that swimming motility is not quorum-sensing-regulated (data not shown). We also tested the strain for twitching motility under various conditions, but were unable to demonstrate this form of motility for strain H111. However, during the course of these experiments we observed that, when medium containing 0.4% agar was supplemented with 0.1% Casamino acids, cells also spread as a thin layer on the top of the agar surface. Microscopic inspection revealed that the cells migrate in a co-ordinated fashion that is characteristic of swarming motility (for reviews see Allison & Hughes, 1991; Harshey, 1994; Eberl *et al.*, 1999). The migration front of the expanding colony is preceded by a visible layer of slime-like material giving the colony a glistening appearance, a phenomenon that is typical for this form of motility. After incubation for 36 h, *B. cepacia* H111 colonized the entire surface of the agar plate (Fig. 6). By contrast, the two *cep* mutants were unable to swarm. Moreover, the mutants were also deficient in the production of extracellular slime. Addition of 200 nM C8-HSL to the medium restored both swarming motility and slime production of mutant H111-I (data not shown). Both phenotypes were also restored when mutant H111-R was complemented with plasmid pBAH27 (*cepR*⁺) (Fig. 6). These results show that swarming motility of *B. cepacia* H111 is under control of the *cep* quorum-sensing system. Previously, it has been shown that AHL-mediated cell-cell communication is also required for swarming motility of *S. liquefaciens* MG1 (Eberl *et al.*, 1996) and *P. aeruginosa* (Köhler *et al.*, 2000). In both bacteria the quorum-sensing systems control the production of biosurfactants, namely rhamnolipids in the case of *P. aeruginosa* and serrawettin W2 in the case of *S. liquefaciens*, which are essential for swarming motility (Ochsner & Reiser, 1995; Lindum *et al.*, 1998; Köhler *et al.*, 2000). A *S. liquefaciens* mutant defective in the synthesis of AHL molecules is unable to swarm unless the medium is supplemented with either AHLs or a compound capable of lowering the surface tension of the medium such as serrawettin W2, surfactin or trace amounts of SDS (Lindum *et al.*, 1998; Eberl *et al.*, 1999). We therefore tested the two *cep* mutants of *B. cepacia* for their ability to swarm on low-agar plates supplemented with either surfactin or serrawettin W2. The two *cep* mutants swarmed on this medium (Fig. 6 and data not shown), suggesting that production of a biosurfactant in *B. cepacia* H111 is controlled by the *cep* quorum-sensing system, which in turn is required for swarming motility of this bacterium.

Swarming motility is not required for biofilm formation

For *Vibrio parahaemolyticus*, it has been demonstrated that swarming plays an important role in attachment and colonization of chitinous shells of crustaceans (Belas & Colwell, 1982). To investigate whether swarming motility is involved in the process of biofilm formation by *B. cepacia*, we tested the wild-type and the

two *cep* mutants for their abilities to form biofilms in minimal medium supplemented with different amounts of surfactin and serrawettin. The presence of high concentrations of the surfactants in the medium completely prevented the cells from attaching to the surface of the microtitre dishes. Importantly, the surfactant concentrations used did not influence the growth rates of the strains in liquid medium (data not shown). At lower concentrations, which were sufficiently high to completely restore swarming behaviour of the mutants, the two surfactants very weakly (less than 10% increase in A_{570}) but reproducibly stimulated biofilm formation by the *cep* mutants while their presence did not affect biofilm formation by the wild-type. However, these results suggest that swarming motility *per se* is unlikely to play a major role in biofilm formation.

DISCUSSION

AHL-dependent communication systems provide bacteria with a regulatory mechanism that enables individual cells to sense their own population density. In response to the size of the population, i.e. when a certain critical mass, the 'quorum' has been attained, cells collectively induce the expression of particular phenotypic traits, which are not observable with individual cells. Hence, quorum-sensing can be viewed as an example of primitive multicellular behaviour. In nature, bacteria are normally associated with surfaces, on which they form highly structured biofilms (Costerton *et al.*, 1995; Davey & O'Toole, 2000; Tolker-Nielsen & Molin, 2000; O'Toole *et al.*, 2000). Bacteria living in biofilms are embedded in a matrix of extracellular polymeric substances and thus cell densities are obviously extremely high in these surface-attached communities. By contrast, bacteria growing planktonically, for example in the water column of aquatic systems, only rarely reach high cell densities. It is therefore conceivable that quorum-sensing is a particularly valuable mechanism for gene regulation in biofilms. In support of this view, it has been recently demonstrated that AHL molecules are present both in natural biofilms growing on submerged stones in a river and in biofilms formed on urethral catheters (McLean *et al.*, 1997; Stickler *et al.*, 1998). Moreover, for *P. aeruginosa* it was shown that the *las* quorum-sensing system is directly involved in the regulation of biofilm formation (Davies *et al.*, 1998). When compared with the wild-type, a *lasI* mutant of *P. aeruginosa* only forms flat and undifferentiated biofilms, suggesting that the *las* system is in some way required for the maturation of biofilms. Importantly, the *lasI* mutant biofilm exhibited greater sensitivity to the biocide SDS in comparison to the wild-type biofilm. The results presented for biofilm formation by *B. cepacia* H111 are fully consistent with this hypothesis. Mutants defective in the *cep* quorum-sensing system form microcolonies on a glass surface that are indistinguishable from those formed by the wild-type, indicating that the early events of biofilm formation are unaffected by the mutations. Similar to the *lasI* mutant

of *P. aeruginosa*, however, these microcolonies are unable to differentiate and thus failed to develop into a mature biofilm.

During an attempt to identify *cep*-regulated factors required for biofilm formation by *B. cepacia*, we noticed that the strain's ability to swarm on suitable surfaces is quorum-sensing-regulated. Swarming motility is an intrinsically surface-dependent mode of translocation, which, to the best of our knowledge, has not been reported earlier for members of the genus *Burkholderia* but has been described for many other bacteria (Allison & Hughes, 1991; Harshey, 1994). Swarming motility of quorum-sensing-defective mutants of *B. cepacia* H111 could be fully restored by supplementing the media with different surfactants. We therefore propose that the *cep* system of *B. cepacia* controls the production of a biosurfactant, which is required for swarming motility of the strain. Noteworthy in this context is the recent finding that another genomovar III *B. cepacia* strain (J2315) produces a lipopeptide of unknown structure which exhibits strong surface-active properties (Hutchinson *et al.*, 1998). Our results are reminiscent of the situation found with *S. liquefaciens* and *P. aeruginosa*. These two bacteria have been demonstrated to employ quorum-sensing systems to control the synthesis of the surface-active compounds serrawettin W2 and rhamnolipids, respectively (Ochsner & Reiser, 1995; Lindum *et al.*, 1998). Since the ability to swarm is strictly dependent on the production of biosurfactants, swarming motility is a quorum-sensing-regulated phenomenon in both bacteria.

The addition of surfactants to the medium, at concentrations sufficiently high to restore swarming motility of the *cep* mutants to the level of the wild-type, only weakly, if at all, increased biofilm formation by the *B. cepacia* H111 *cep* mutants. This suggests that locomotion via swarming motility is not required for biofilm formation. On the other hand, the *cep*-regulated production of the biosurfactant itself may affect biofilm formation as previous results have demonstrated that various surface-active compounds have the capability of regulating the attachment and detachment of bacteria to and from surfaces (Rosenberg & Ron, 1999; Ahimou *et al.*, 2000). There are several possible explanations as to why our attempts to substitute the missing biosurfactant with surfactin, serrawettin W2 or SDS failed to restore biofilm formation by the *cep* mutants: (i) the physical properties of the surfactants used and the one produced by the strain are substantially different, (ii) production of the surfactant has to follow a specific temporal and/or spatial expression pattern within the biofilm, or (iii) other *cep*-regulated, as yet unidentified, factors may be required for biofilm formation. Work currently under way aims to test these possibilities.

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