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 & Höiby, 1993; Govan & Deretic, 1996; Tümmler & Kiewitz, 1999). During chronic infection, *P. aeruginosa* produces copious amounts of alginate, which forms a matrix completely embedding...
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* exproducts 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 (genovar 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, plasmin 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 & Maaloe, 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-L, 200 mM C8-HSL and/or 200 mM C6-HSL were added. Growth of liquid cultures was monitored spectrophotometrically by an Ultrascan 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 Taq 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′-GGGCTTCAACCTG-ACAAGTATGACACGG-3′) and cepR-OV (5′-GGGCTTACCGGATGACATGGGAGAAGGC-3′) (KpnI restriction sites are underlined). Following digestion with KpnI, the PCR fragments were inserted into the broad-host-range vector pBRR1MCs-5 cut with the same enzyme. The plasmid
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_{fluorescent protein} (GFP). This was accomplished by the insertion of a P_{fluorescent protein} (GFP) using the suicide construct pMH94 (M. Hentzer & M. R. Parsek, unpublished results). Plasmid pMH94 was delivered using the helper plasmid in triparental matings de Lorenzo & Timmis (1994)

AHLs were extracted twice with dichloromethane (250:100 super-
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**Table 1. Bacterial strains and plasmids used in this study**

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<th>Strain or plasmid</th>
<th>Description</th>
<th>Source or reference</th>
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<td>pGEM-Zf(+)</td>
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<td>pMH94</td>
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<tr>
<td>pH600</td>
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<tr>
<td>pTTmini-Tn5Km2-luxCDABE</td>
<td>Km', delivery vector for mini-Tn5Km2-luxCDABE</td>
<td>This study</td>
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**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 polyacrylatic 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 pSB430 (Winson et al., 1998b). This sensor plasmid contains the Photobacterium fisheri luxR gene together with the luxl promoter region as a transcriptional fusion to the bioluminescence genes luxCDABE of Photorhabdus luminescens. The quorum-sensing system of Photobacterium fisheri relies on N-[3-(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(pSB430) 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 (Rf values) relative to those for the synthetic AHL standards.

For quantification of AHL signal molecules, 100 µl of filter-
sterilized supernatants of cultures grown in LB medium to an OD600 of 3.0 were added to 100 µl 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).

**Enzyme and siderophore production.** Enzyme 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 (Knittel Glaeser). Overnight cultures in ABG medium were subcultured to an OD600 of 0.7 before dilution in 0.9% NaCl to an OD600 of 0.1. Aliquots (300 µl) 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 × 1.32-0.6 oil objective. For statistical evaluation of biofilm structures, a 40 × 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, substrate 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.

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**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 palindromic sequence in the cepl 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/loxP element in *B. cepacia* m64 is indicated by a triangle. The positions of the npt cassettes in the cepl mutant H111-I and the cepR mutant H111-R are indicated by circles.

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The primer pair intercep-f-Eco (5'-GCCGATCCGAGATCTCGGG-3') and intercep-r-Sac (5'-CGGATCCGAGATCTCGGG-3') and a 760 bp SplI–HindIII fragment containing the 3′ region of the cepR gene using the primer pair cepR-f-Sph (5′-ACATGATCAGCCTCGGATCTGTTAATACGTCG-3') and cepR-r-Hind (5′-CTCGAGATCTCGGCTCGGATCTGTTAATACGTCG-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 17 kb BamHI 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 tetacycline. To screen for gene replacement mutants, Kan' 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 cepl 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 integeregic region plus 100 bp of cepR was amplified with the primer pair igR-f-Eco (5′-GGATCCCGAGATCCGAGATCTCGGG-3') and igR-r-Sac (5′-CGGATCCGAGATCTCGGG-3'), and a 650 bp BamHI–HindIII fragment containing the 5′ region of cepR was amplified using the primer pair cepl-f-Bam (5′-GGATCCGAGATCTCGGG-3') and cepR-Hind (5′-CCTGACGTTCGAGATCTCGGG-3'). The final gene replacement construct was designated pAG and the respective *B. cepacia* H111 cepl 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 Glaeser). Overnight cultures in ABG medium were subcultured to an OD600 of 0.7 before dilution in 0.9% NaCl to an OD600 of 0.1. Aliquots (300 µl) 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.

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

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>H111</th>
<th>H111-R (pBAH27)</th>
<th>H111-I</th>
<th>H111-I+ AHD</th>
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<td>AHL</td>
<td>+</td>
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<td>+</td>
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<td>Protease</td>
<td>+</td>
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<td>Lipase</td>
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<td>+</td>
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<tr>
<td>Siderophore</td>
<td>+ (+)</td>
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<td>Swarming</td>
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<td>Swimming</td>
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The suicide vector pUT (de Lorenzo & Timmis, 1994) was used to deliver the hybrid transposon mini-Tn5 Km2-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 mutants 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...
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-IMCS-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<sup>+</sup>) 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<sup>+</sup>). 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 analyze 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<sub>A</sub><sup>16</sup>/40<sup>3</sup>gfpr-T0-T1 transposon cassette (Andersen et al., 1998) randomly...
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; Watanuki & 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 cepL 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.
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 swimming 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 swimming 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 swimming motility and slime production of mutant H111-R (data not shown). Both phenotypes were also restored when mutant H111-R was complemented with plasmid pBAH27 (*cepR*) (Fig. 6). These results show that swimming 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 swimming 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 swimming 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 swimming motility of this bacterium.

**Swimming motility is not required for biofilm formation**

For *Vibrio parahaemolyticus*, it has been demonstrated that swimming plays an important role in attachment and colonization of chitinaceous shells of crustaceans (Belas & Colwell, 1982). To investigate whether swimming 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 and in 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.

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