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The *cep* Quorum-Sensing System of *Burkholderia cepacia* H111 Controls Biofilm Formation and Production of Virulence Factors

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Introduction

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 (Govan & Deretic, 1996). 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. This hypothesis was recently corroborated through profiling of AHL signal molecules (Singh *et al.*, 2000). *Burkholderia cepacia* has been recognised as another important pathogen in patients with CF. Infection with *B. cepacia* often occurs in patients who are already colonised with *P. aeruginosa*.

In both *P. aeruginosa* and *B. cepacia* 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. Most 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 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).

Results

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

In order to investigate the role of the *cepIR* genes for biofilm formation we constructed site-directed insertion mutations in the two genes by using a gene replacement method (Hoang *et al.*, 1998). 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 (data not shown). 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 pBBR1MCS-5 (Kovach *et al.*, 1995).

B. cepacia produces different siderophores and a number of exoenzymes that are thought to be pathogenesis factors in humans as well as in plants. 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 1. 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*$^+$). 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 (O'Toole & Kolter, 1998). Both mutants were found to be defective in biofilm formation (Fig. 1). However, addition of 200 nM C8-HSL to the medium completely restored biofilm formation of mutant H111-I. Likewise, H111-R harbouring plasmid pBAH27 formed wild type biofilms.

To further analyse the role of the *cep* system for biofilm formation we employed artificial flow cells, which allow to follow biofilm development 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 (CLSM) the three-dimensional structure of the biofilm can be reconstructed. For this analysis it was necessary to

Table 1. 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 (Winson *et al.*, 1998). Synthesis of extracellular hydrolytic enzymes and siderophore activities were assessed by streaking strains on appropriate indicator plates. Swimming and swarming motility was tested on media solidified with 0.3 % and 0.4 % agar, respectively. The wild type H111, the *cepR* mutant H111-R, the complemented *cepR* mutant H111-R (pBAH27), and the *cepI* mutant in the presence or of C8-HSL (200 nM) were investigated. +, activity exhibited by the wild type; (+) significantly reduced activity; -, no detectable activity; n.a., not applicable.

Phenotype	wild type	H111-R	H111-R (pBAH27)	H111-I	H111-I + AHL
AHL	+	-	+	-	n.a.
Protease	+	-	+	-	+
Chitinase	+	(+)	+	(+)	+
Lipase	+	+	+	+	+
Siderophore	+	(+)	+	(+)	+
Swarming	+	-	+	-	+
Swimming	+	+	+	+	+

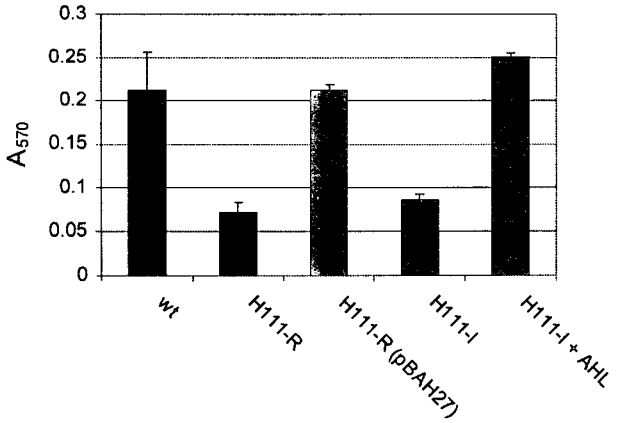

Fig. 1. The *cep* quorum-sensing system controls biofilm formation of *B. cepacia* H111. Strains were grown in AB minimal medium supplemented with 10 mM citrate in the wells of polypropylene microtitre dishes. After incubation for 48 hours at 30°C planktonic cells were removed and attached cells were then stained with crystal violet. Biofilms formed by the wild type H111, the *cepR* mutant H111-R, the complemented *cepR* mutant H111-R (pBAH27), and the *cepI* mutant in the presence or absence of C8-HSL (200 nM) are shown. Error bars represent the standard deviation of the mean for six independent wells.

tag *B. cepacia* H111 and the two mutants H111-I and H111-R with the green fluorescent protein (Gfp). This was accomplished by inserting a $P_{A1/04/03}$-*gfp*-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).

Parallel flow chambers were inoculated with each of the three Gfp-tagged strains and biofilm development was monitored on a daily basis for seven days. Visual inspection of CLSM images revealed that the biofilms formed by the two mutants did not only differ in their substratum coverage and thickness, as was already anticipated from the microtitre plate assays, but also exhibited strikingly different structures (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 already within 24 hours, mutant biofilms were arrested in the microcolony stage and never colonised the entire surface during the course of the experiment.

In order to more accurately describe the differences of 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 have chosen the following: biomass, average 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 hours for 7 days and these were analysed with COMSTAT (Heydorn *et al.*, 2000). In full agreement with the visual impression, the coefficients for average thickness and for biomass were greatly reduced for the *cep* mutants when compared with the wild type (Table 2). The two mutants colonise 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 for the variance of biofilm thickness and the higher values of this coefficient for mutant

Table 2. Quantification of biofilm structures. Biofilms of the wild type H111, and the *cepI* mutant H111-I in the presence or absence of 200 nM C8-HSL were grown in artificial flow cells. CLSM pictures were taken daily for one week and these pictures were analysed by the computer program COMSTAT (Heydorn *et al.*, 2000). Parameters calculated are biomass, average biofilm thickness, substratum coverage, and roughness coefficient. Mean values for seven independent CLSM pictures are shown with standard deviations.

Biomass (m3/ m2)			
Day	wild type	H111-I	H111-I + AHL
1	0.48 +/- 0.29	0.36 +/- 0.27	1.04 +/- 0.24
2	7.40 +/- 2.1	2.99 +/- 1.7	7.14 +/- 0.82
3	9.57 +/- 2.3	3.14 +/- 0.79	10.02 +/- 1.9
4	13.83 +/- 3.3	3.03+/- 1.1	10.02 +/- 1.7
5	18.56 +/- 3.0	2.98 +/- 1.7	16.93 +/- 1.9
6	14.06 +/- 2.6	3.15 +/- 1.0	15.20 +/- 2.1
7	21.48 +/- 7.7	3.11 +/- 1.0	14.58 +/- 0.98
Substratum coverage			
Day	wild type	H111-I	H111-I + AHL
1	0.11 +/- 0.077	0.097 +/- 0.067	0.27 +/- 0.06
2	0.78 +/- 0.10	0.46 +/- 0.25	0.56 +/- 0.18
3	0.77 +/- 0.17	0.56 +/- 0.21	0.77 +/- 0.16
4	0.79 +/- 0.11	0.51 +/- 0.11	0.87 +/- 0.090
5	0.86 +/- 0.088	0.45 +/- 0.18	0.90 +/- 0.074
6	0.82 +/- 0.081	0.52 +/- 0.15	0.90 +/- 0.072
7	0.89 +/- 0.067	0.45 +/- 0.15	0.90 +/- 0.071
Roughness			
Day	wild type	H111-I	H111-I + AHL
1	1.86 +/- 0.084	1.94 +/- 0.036	1.77 +/- 0.083
2	0.68 +/- 0.18	1.27 +/- 0.033	0.52 +/- 0.080
3	0.56 +/- 0.064	1.27 +/- 0.25	0.39 +/- 0.11
4	0.46 +/- 0.089	1.31 +/- 0.29	0.24 +/- 0.030
5	0.34 +/- 0.071	1.33 +/- 0.39	0.22 +/- 0.053
6	0.47 +/- 0.085	1.27 +/- 0.20	0.29 +/- 0.060
7	0.34 +/- 0.18	1.13 +/- 0.20	0.34 +/- 0.060
Average Thickness (m)			
Day	wild type	H111-I	H111-I + AHL
1	0.21 +/- 0.14	0.10 +/- 0.066	0.35 +/- 0.14
2	6.78 +/- 2.3	2.26 +/- 1.4	7.47 +/- 0.89
3	10.02 +/- 2.9	2.05 +/- 0.72	10.76 +/- 2.2
4	16.21 +/- 4.2	2.11 +/- 1.1	16.82 +/- 1.6
5	22.15 +/- 3.8	2.40 +/- 1.9	19.06 +/- 1.9
6	17.75 +/- 3.5	2.35 +/- 1.1	17.94+/- 2.3
7	28.60 +/- 11.9	2.43 +/- 1.1	17.16 +/- 2.3

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 (Table 2). Likewise, strain H111-R harbouring plasmid pBAH27 (*cepR*$^+$) forms a biofilm with a typical wild type structure (data not shown).

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.

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. 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, e.g. 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. 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 sodium dodecyl sulfate in comparison to the wild type biofilm. The results presented for biofilm formation of *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.

In an attempt to identify *cep*-regulated factors required for biofilm formation of *B. cepacia* we noticed that the strain ability to swarm on suitable surfaces is quorum-sensing regulated (data not shown). 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. 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.. Our results are reminiscent to 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).

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 of 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). 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 of 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, yet unidentified, factors may be required for biofilm formation. Work currently under way aims at testing these possibilities.

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References

Andersen, J. B., Sternberg, C., Poulsen, L. K., Petersen Bj n, S., Givskov, M. & Molin, S. (1998). New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. *Appl Environ Microbiol* **64**, 2240-2246.

Christensen, B. B., Sternberg, C., Andersen, J. B., Palmer, R. J., Nielsen, A. T., Givskov, M. & Molin, S. (1999). Molecular tools for the study of biofilm physiology. *Methods Enzymol* 310, 20-42.

Davies, D. G., Parsek, M. R., Pearson, J. P., Iglewski, B. H., Costerton, J. W. & Greenberg, E. P. (1998). The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* 280, 295-298.

Govan, J. R. W. & Deretic, V. (1996). Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiol Rev* 60, 539-574.

Heydorn, A., Nielsen, A. T., Hentzer, M., Sternberg, C., Givskov, M., Ersb l, B. K. & Molin, S. (2000). Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology* 146, 2395-2407.

Hoang, T. T., Karkhoff-Schweizer, R. R., Kutchma, A. J. & Schweizer, H. P. (1998). A broad-host range Flp-*FRT* recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* 212, 77-86.

Köhler, T., Curty, L. K., Barja, F., van Delden, C. & Pechère J.C. (2000). Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. *J Bacteriol* 182, 5990-5996.

Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M. & Peterson, K. M. (1995). Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* 166, 175-176.

Lewenza, S., Conway, B., Greenberg, E. P. & Sokol, P. A. (1999). Quorum sensing in *Burkholderia cepacia*: Identification of the LuxRI homologs CepRI. *J Bacteriol* 181, 748-765.

Lindum, P. W., Anthoni, U., Christophersen, C., Eberl, L., Molin, S. & Givskov, M. (1998). *N*-Acyl-L-homoserine lactone autoinducers control production of an extracellular lipopeptide biosurfactant required for swarming motility of *Serratia liquefaciens* MG1. *J Bacteriol* 180, 6384-6388.

Ochsner, U. A. & Reiser, J. (1995). Autoinducer-mediated regulation of rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* 92, 6424-6428.

O'Toole, G. A. & Kolter, R. (1998). Initiation of biofilm formation in *Pseudomonas fluorescencs* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Mol Microbiol* **28**, 449-461.

Rosenberg, E. & Ron, E. Z. (1999). High- and low-molecular mass microbial surfactants. *Appl Microbiol Biotechnol* **52**, 154-162.

Sanchez-Romero J. M., Diaz-Orejas R. & de Lorenzo V. (1998). Resistance to tellurite as a selection marker for genetic manipulations of *Pseudomonas* strains. *Appl Environ Microbiol* **64**, 4040-4046.

Singh, P. K., Schaefer, A. L., Parsek, M. R., Moninger T. O., Welsh, M. J. & Greenberg, E. P. (2000). Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature* 407, 762-764.

Winson, M. K., Swift, S., Fish, L., Throup, J. P., J gensen, F., Chhabra, S. R., Bycroft, B. W., Williams, P. & Stewart, G. S. A. B. (1998). Construction and analysis of *luxCDABE*-based plasmid sensors for investigating *N*-acyl homoserine lactone-mediated quorum sensing. *FEMS Microbiol Let* 163, 185-192.