The catabolite repression control protein Crc plays a role in the development of antimicrobial-tolerant subpopulations in *Pseudomonas aeruginosa* biofilms

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Bacteria form complex surface-attached biofilm communities in nature. Biofilm cells differentiate into subpopulations which display tolerance towards antimicrobial agents. However, the signal transduction pathways regulating subpopulation differentiation in biofilms are largely unelucidated. In the present study, we show that the catabolite repression control protein Crc regulates the metabolic state of *Pseudomonas aeruginosa* cells in biofilms, and plays an important role in the development of antimicrobial-tolerant subpopulations in *P. aeruginosa* biofilms.

INTRODUCTION

Bacteria tend to form surfaced-attached biofilm communities that consist of microcolonies embedded in self-produced extracellular polymer substances (Costerton *et al.*, 1995). When biofilms form inside our bodies or at indwelling medical devices they may cause a wide range of health problems due to their resistance towards conventional antimicrobial agents (Stewart & Costerton, 2001). Understanding the physiology of biofilms is crucial for the development of strategies to control them.

The opportunistic pathogen *Pseudomonas aeruginosa* is a model organism for bacterial biofilm research (Harmsen *et al.*, 2010). *P. aeruginosa* biofilm development is a dynamic process that involves motility, iron signalling, quorum sensing, biosurfactant production, and exopolymer synthesis (Allesen-Holm *et al.*, 2006; Pamp & Tolker-Nielsen, 2007; Yang *et al.*, 2007, 2009, 2011). In the flow-chamber model, *P. aeruginosa* differentiates into at least two subpopulations during biofilm formation (Klausen *et al.*, 2003a), and these subpopulations have been shown to confer tolerance to various antimicrobial agents, partly due to their differential physiological activity (Pamp *et al.*, 2008). Conventional antimicrobial agents that are known to interfere with fundamental physiological processes of bacterial cells, such as replication (e.g. ciprofloxacin) or translation (e.g. tetracycline), have been found to specifically kill the metabolically active cells in the surface layer of biofilms, whereas cells with low metabolic activity inside the biofilms survive the treatment (Pamp *et al.*, 2008). In contrast, antimicrobial agents that interfere with bacterial membrane structure and function, such as colistin, EDTA and SDS, have been found to kill cells with low metabolic activity inside biofilms, whereas cells with high metabolic activity in the surface layer of the biofilm survive the treatment due to their ability to induce resistance mechanisms (Pamp *et al.*, 2008; Chiang *et al.*, 2012).

The existence of subpopulations with different physiological activities in biofilms is thought to be caused mainly by nutrient gradients (e.g. Stewart & Franklin, 2008), but the underlying mechanism of differentiation in metabolic status of the cells in *P. aeruginosa* biofilms remains unclear. The catabolite repression control (Crc) protein is a post-transcriptional global regulator of carbon metabolism in *Pseudomonas* (MacGregor *et al.*, 1991; Morales *et al.*, 2004; Moreno *et al.*, 2007). It has been shown to inhibit the expression of several genes involved in the transport and catabolism of sugars (Collier *et al.*, 1996; Hester *et al.*, 2000; Moreno *et al.*, 2009). Recently, the *P. aeruginosa* Crc protein has been linked to antibiotic resistance (Linares *et al.*, 2010). Evidence has been presented that a *P. aeruginosa* crc mutant is more susceptible to β-lactams, aminoglycosides, fosfomycin and rifampicin than the corresponding wild-type strain.

Abbreviation: CLSM, confocal laser scanning microscopy.
Because Crc is essential for fine tuning the metabolism of *P. aeruginosa* cells, we hypothesized that it is involved in downregulation of the metabolic activity of the bacteria inside *P. aeruginosa* biofilms in response to nutrient limitation. In agreement with this hypothesis we provide evidence in the present study that a *P. aeruginosa* crc mutant biofilm has a higher metabolic activity than the corresponding wild-type biofilm. Moreover, we show that *P. aeruginosa* crc mutant biofilm is more susceptible to ciprofloxacin and more tolerant to colistin than the wild-type biofilm.

**METHODS**

**Bacterial strains and growth conditions.** *P. aeruginosa* PA14 (O’Toole & Kolter, 1998) was used as the wild-type strain in this study. The Crc-defective PA14Δcrc mutant (O’Toole et al., 2000) and its complemented PA14Δcrc+ mutant (O’Toole et al., 2000) were kindly provided by Dr George A. O’Toole (Dartmouth Medical School, Hanover, USA). *Escherichia coli* strain DH5α was used for standard DNA manipulations. The gfp-tagged, cfp-tagged (cyan fluorescent protein) and yfp-tagged (yellow fluorescent protein) *P. aeruginosa* strains were constructed by insertion of mini-Tn7-cEFP-Strep, mini-Tn7-cEFP-Strep and mini-Tn7-eYFP-Strep, respectively, as described by Klausen et al. (2003b). Luria–Bertani medium (Bertani, 1951) was used to cultivate *E. coli* strains. Batch cultivation of *P. aeruginosa* was carried out at 37 °C in AB minimal medium (Clark & Maaløe, 1967) supplemented with 30 mg glucose l⁻¹. For plasmid maintenance in *E. coli*, the medium was supplemented with 100 μg ampicillin (Ap) ml⁻¹, 15 μg gentamicin (Gm) ml⁻¹, 50 μg streptomycin (Strep) ml⁻¹ or 8 μg chloramphenicol (Cm) ml⁻¹. For marker selection in *P. aeruginosa*, 60 μg Gm ml⁻¹ and 100 μg Strep ml⁻¹ were used, as appropriate.

**Resazurin assay.** The non-toxic oxidation–reduction-sensitive dye resazurin is widely used as an indicator of the metabolic activity of eukaryotic and prokaryotic cells (Sarker et al., 2007). Resazurin is blue and nonfluorescent, but it becomes pink and highly red fluorescent after it is reduced to resorufin by cells. The non-toxic oxidation–reduction-sensitive dye resazurin was used to cultivate *P. aeruginosa* biofilms in flow chambers, the blue resazurin was reduced to pink resorufin at a faster rate close to the biofilm (our unpublished observations). Thus, resazurin was used as an indicator of the metabolic activity of *P. aeruginosa* biofilms in flow chambers. The oxidation of resazurin by stationary phase cells was monitored at 540 nm excitation wavelength and 590 nm emission wavelength.

**Biofilm assay.** *P. aeruginosa* biofilms were cultivated at 30 °C in flow chambers irrigated with FAB medium (Heydorn et al., 2000) supplemented with 0.3 mM glucose, as previously reported (Sternberg & Tolker-Nielsen, 2006). Biofilm images were taken by confocal laser scanning microscopy (CLSM) using a Zeiss LSM 510 microscope (Carl Zeiss). Vertical cross section images were generated using the IMARIS software package (Bitplane).

To measure the metabolic rate of *P. aeruginosa* biofilms, the biofilms grown in flow chambers were first washed with 200 μFAB medium to remove planktonic cells. Then, 200 μFAB medium containing 25 μM resazurin was injected into the flow chambers. The colour change of the flow chambers was recorded by the use of a digital camera at different time points.

**Antimicrobial treatment.** Biofilm tolerance to ciprofloxacin or colistin was assessed by irrigating 4-day-old flow chamber-grown *P. aeruginosa* biofilms with medium containing 20 μg ciprofloxacin or 20 μg colistin ml⁻¹ for 24 h, followed by staining of the dead cells with 1 μM propidium iodide, and CLSM image acquisition.

**RESULTS**

**Crc affects early stage but not late stage biofilm development by *P. aeruginosa***

For our assessment of metabolic activity and antimicrobial tolerance in flow-chamber biofilms, it was important to grow the *P. aeruginosa* PA14, PA14Δcrc and PA14Δcrc/ pcrc+ strains in the flow chambers under conditions where they formed biofilms with similar structure and biomass. After 1 day of growth on FAB glucose medium, the PA14 wild-type formed biofilm consisting of a thin layer of cells (Fig. 1, left column), but there was only a very limited amount of PA14Δcrc mutant cells attached to the substratum (Fig. 1, centre column), whereas the complemented mutant PA14Δcrc/pcrc+ formed biofilm with some small microcolony structures similar to the wild-type (Fig. 1, right column). However, after 4 days of cultivation, all three *P. aeruginosa* strains had formed thick biofilms with microcolony structures in the flow chambers (Fig. 1, lower row). These experiments suggest that the crc mutant has a defect in initial biofilm development, in agreement with earlier studies (O’Toole et al., 2000), but also that the *P. aeruginosa* crc mutant formed biofilms similar to the wild-type at later stages. After 4 days of cultivation the biofilms in the flow chambers approached a steady-state level, and it appears that at this time point the difference in biofilm biomass between the wild-type and crc mutant levels out.

**Crc regulates the metabolic status of cells from *P. aeruginosa* biofilms and from the stationary growth phase**

We used the redox dye resazurin to examine the metabolic state of the bacteria in 4 day-old biofilms formed by the *P. aeruginosa* PA14 wild-type, the PA14Δcrc mutant and the PA14Δcrc/pcrc+ complemented mutant. The arrows in Fig. 2 indicate the direction of medium flow and resazurin injection in the flow chambers. After injection into the flow chambers, the blue resazurin was reduced to pink resorufin within a short period of time. Biofilm close to the inlet of the flow chamber had higher metabolic activity than biofilm close to the outlet of the flow chamber due to a nutrient gradient arising in the segment of the laminar flow close to the biofilm (our unpublished observations). Thus, resazurin was reduced to resorufin at a faster rate close to the inlet of the flow chambers than close to the outlet, reflected in a gradient of resazurin reduction from the inlet to the outlet of the flow chambers (Fig. 2). Twenty minutes after resazurin injection, a smaller fraction of the redox dye was reduced to pink resorufin by biofilms of PA14 and PA14Δcrc/pcrc+ than by biofilms of PA14 Δcrc (Fig. 2), indicating that PA14Δcrc mutant biofilms have increased metabolic activity, which supports the hypothesis that Crc

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has a role in reducing the metabolic activity of the bacteria in the inner part of *P. aeruginosa* biofilms.

The bacteria in the inner part of *P. aeruginosa* biofilms show some similarity to stationary phase cells from planktonic cultures. We investigated the impact of Crc on the metabolic state of *P. aeruginosa* cells from the late stationary phase of planktonic cultures. As shown in Fig. 3, resazurin was reduced at a faster rate by the PA14Δcrc mutant strain than by the PA14 wild-type and the PA14Δcrc/pcrc+ complemented strain, indicating that stationary phase cells of the PA14Δcrc mutant have higher metabolic activity than stationary phase cells of the PA14 wild-type.

**Crc plays a role in the development of antimicrobial-tolerant subpopulations in mature *P. aeruginosa* biofilms**

Based on the results described above we employed 4 day-old *P. aeruginosa* PA14, PA14Δcrc and PA14Δcrc/pcrc+ biofilms for our investigation of the role of crc in antimicrobial tolerance in *P. aeruginosa* biofilms. We have previously provided evidence that the cells in the outer subpopulation in *P. aeruginosa* flow-chamber biofilms are metabolically active, whereas the cells in the inner subpopulation display low metabolic activity (Pamp *et al.*, 2008). Because ciprofloxacin was shown specifically to kill the metabolically active cells in these biofilms, whereas colistin was shown specifically to kill the metabolically inactive cells (Pamp *et al.*, 2008), we challenged biofilms with these two antimicrobial agents in the present study. After ciprofloxacin treatment, most of the biofilm formed by the PA14Δcrc mutant was killed, whereas only the surface subpopulation of the biofilm formed by *P. aeruginosa* PA14 and PA14Δcrc/pcrc+ was killed (Fig. 4). In contrast, after colistin treatment the PA14Δcrc mutant biofilm consisted of live cells interspersed...
with a minor fraction of dead cells, whereas only the surface subpopulation of the biofilms formed by the *P. aeruginosa* PA14 and PA14Δcrc/pcrc+ strains was alive (Fig. 5). Spatially confined red and green subpopulations were observed in the biofilms formed by the PA14 wild-type and PA14Δcrc/pcrc+ complemented strain, but not in the biofilm formed by the PA14Δcrc mutant (Fig. 5).

**DISCUSSION**

Biofilms are well known to contain microbial cells in a wide range of physiological states (Stewart & Franklin, 2008). Substrate gradients caused by nutrient consumption by the bacteria located at the periphery of the biofilm are a major contributor to the physiological heterogeneity of biofilms (Xu et al., 1998; Sternberg et al., 1999; Pamp et al., 2008). It may be assumed that target inactivity enables growth-arrested bacteria inside biofilms to tolerate antimicrobial agents that are known to interfere with fundamental bacterial physiological processes. Thus, if targets are inactive, e.g. quinolones such as ciprofloxacin may not generate DNA breaks, aminoglycosides may not cause mistranslation, and β-lactams may not cause peptidoglycan breaks. However, growth arrest in response to nutrient limitation is likely to occur as a controlled process that ensures survival of the bacteria. Nutrient limitation-induced antimicrobial tolerance may therefore depend on adaptive starvation responses instead of simply target inactivity. In the present study we show that the catabolite repression control protein Crc plays a role in down-regulating the metabolic activity of cells in biofilms and in stationary phase planktonic cultures. In accordance with this, we show that ciprofloxacin, which is known specifically to kill the metabolically active cells in *P. aeruginosa* biofilms (Pamp et al., 2008), kills the majority of the cells in a *P. aeruginosa* crc mutant biofilm, whereas it only kills the surface subpopulation in wild-type biofilms.
Conversely, we show that colistin, which is known specifically to kill the metabolically inactive cells in *P. aeruginosa* biofilms (Pamp et al., 2008), fails to kill the majority of the cells in a *P. aeruginosa* *crc* mutant biofilm, whereas it kills the inner subpopulation in wild-type biofilms. Our results thus indicate that the metabolically inactive subpopulation in *P. aeruginosa* biofilms does not arise solely due to nutrient limitation. It appears that the cells can sense limited nutrient conditions and then differentiate into metabolically inactive subpopulations via Crc, putatively as part of a survival strategy.

In agreement with our study, a recent study has documented that antibiotic tolerance of nutrient-limited and biofilm *P. aeruginosa* is mediated by active responses to starvation, rather than by the passive effects of growth arrest (Nguyen et al., 2011). Evidence was provided that the protective mechanism is controlled by the stringent response, and the experiments linked stringent response-mediated antimicrobial tolerance to reduced levels of oxidant stress in the bacteria. Inactivation of the stringent response mechanism was shown to sensitize biofilms grown in microtitre wells by several orders of magnitude to four different classes of antibiotics. However, unlike our study, inactivation of the starvation response did not affect antimicrobial tolerance of flow-channel biofilms in the study of Nguyen et al. (2011). Moreover, unlike our *P. aeruginosa* *crc* mutant biofilms, which showed increased tolerance to colistin, Nguyen et al. (2011) found that a *P. aeruginosa* stringent response mutant biofilm displayed reduced tolerance to colistin.

Identification of antimicrobial tolerance mechanisms is important for devising new therapeutic strategies against microbial infections. In the present report we have provided evidence that Crc-mediated physiological adaptations to nutrient limitation are involved in the development of antimicrobial-tolerant subpopulations in *P. aeruginosa* biofilms. This finding suggests that interference with starvation-response mechanisms may enhance the ability of conventional antibiotics to eradicate biofilm infections.

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