Effects of iron on DNA release and biofilm development by *Pseudomonas aeruginosa*

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Extracellular DNA is one of the major matrix components in *Pseudomonas aeruginosa* biofilms. It functions as an intercellular connector and plays a role in stabilization of the biofilms. Evidence that DNA release in *P. aeruginosa* PAO1 biofilms is controlled by the *las-rhl* and *pqs* quorum-sensing systems has been previously presented. This paper provides evidence that DNA release in *P. aeruginosa* PAO1 biofilms is also under iron regulation. Experiments involving cultivation of *P. aeruginosa* in microtitre trays suggested that *pqs* expression, DNA release and biofilm formation were favoured in media with low iron concentrations (5 μM FeCl_3), and decreased with increasing iron concentrations. Experiments involving cultivation of *P. aeruginosa* in a flow-chamber system suggested that a high level of iron (100 μM FeCl_3) in the medium suppressed DNA release, structural biofilm development, and the development of subpopulations with increased tolerance toward antimicrobial compounds. Experiments with *P. aeruginosa* strains harbouring fluorescent reporters suggested that expression of the *pqs* operon was induced in particular subpopulations of the biofilm cells under low-iron conditions (1 μM FeCl_3), but repressed in the biofilm cells under high-iron conditions (100 μM FeCl_3).

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INTRODUCTION

Biofilms are sessile populations of micro-organisms that are surrounded by the slime they produce, and may be attached to an inert or living surface. Biofilm formation is described as a developmental sequence that varies to some extent between bacterial species but often results in the formation of mature microbial communities with tower- or mushroom-shaped microcolonies (Davey & O'Toole, 2000; Sauer *et al.*, 2002; Klausen *et al.*, 2003a). The bacteria in biofilms in medical settings often display increased resistance toward host immune responses and enhanced tolerance toward antibiotic treatment (Costerton *et al.*, 1999). Knowledge about the molecular mechanisms that are involved in biofilm development, and the factors that lead to the formation of resistant subpopulations, may be useful for creating strategies to control biofilm formation and eradicate persistent infections.

One of the most distinctive features of biofilms is the extracellular polymeric substances that surround the bacteria and constitute the biofilm matrix. The composition of biofilm matrices varies depending upon the bacterial species and the environmental conditions, but in general they contain components such as polysaccharides, proteins

and extracellular DNA (Sutherland, 2001; Whitchurch *et al.*, 2002).

Evidence for a role of extracellular DNA as matrix component in *Pseudomonas aeruginosa* biofilms has been presented both for the *P. aeruginosa* PAO1 reference strain and for clinical *P. aeruginosa* isolates (Whitchurch *et al.*, 2002; Nemoto *et al.*, 2003). *P. aeruginosa* PAO1 biofilm development in the wells of microtitre plates was attenuated by the presence of DNase I, and biofilm formation by *P. aeruginosa* PAO1 in flow-chambers was almost absent when the flow-chambers were irrigated with medium containing DNase I (Whitchurch *et al.*, 2002). In addition, young *P. aeruginosa* PAO1 biofilms that had been grown in flow-chambers irrigated with DNase-free medium dispersed rapidly after addition of DNase I to the flowing medium, whereas mature *P. aeruginosa* PAO1 biofilms were not dispersed by DNase I treatment, suggesting that components other than extracellular DNA stabilize mature *P. aeruginosa* PAO1 biofilms (Whitchurch *et al.*, 2002). Matsukawa & Greenberg (2004) investigated the composition of the extracellular matrix of mature *P. aeruginosa* PAO1 biofilms and found that extracellular DNA was by far the most abundant polymer, although exopolysaccharide appeared to be the most critical structural matrix component. In contrast to the finding that extracellular DNA may not be the primary cell-to-cell interconnecting compound in mature *P. aeruginosa* PAO1 biofilms, Nemoto

Abbreviations: AHL, *N*-acylhomoserine lactone; CLSM, confocal laser scanning microscope/microscopy; DDAO, 7-hydroxy-9*H*-(1,3-dichloro-9,9-dimethylacridin-2-one); PQS, *Pseudomonas* quinolone signal.

et al. (2003) found that mature biofilms formed by four different clinical *P. aeruginosa* isolates could be dispersed by DNase treatment, suggesting that extracellular DNA is the critical matrix component in mature biofilms formed by these *P. aeruginosa* strains.

Quorum sensing, a mechanism by which bacteria can monitor their cell population density through the extracellular accumulation of signalling molecules, has been shown to play a role in structural development and stabilization of *P. aeruginosa* biofilms (Davies *et al.*, 1998). *P. aeruginosa* employs the three interconnected quorum-sensing systems *las*, *rhl* and *pqs* to control expression of a wide range of virulence factors (Passador *et al.*, 1993; Latifi *et al.*, 1995, 1996; Pesci *et al.*, 1999). The major signal molecules involved are 3-oxo-C12-homoserine lactone (3-oxo-C12-HSL) in the case of the *las* system, C4-HSL in the case of the *rhl* system, and 2-heptyl-3-hydroxy-4-quinolone, designated the *Pseudomonas* quinolone signal (PQS), in the case of the *pqs* system (Latifi *et al.*, 1995, 1996; Pesci *et al.*, 1999). Synthesis of PQS depends on several putative enzymes encoded by the *pqsABCDE*, *pqsL*, *phnAB* and *pqsH* genes, as well as on a LysR-type regulator encoded by the *pqsR* gene (also known as *mvfR*) (Cao *et al.*, 2001; Gallagher *et al.*, 2002; D'Argenio *et al.*, 2002). Evidence has been presented that the quorum-sensing systems play a role in the formation of extracellular DNA in *P. aeruginosa* biofilms (Allesen-Holm *et al.*, 2006). Quorum-sensing-regulated DNA release from *P. aeruginosa* populations presumably involves lysis of a subpopulation of the cells (or lysis of DNA-containing vesicles), as experiments with *lacZ*-containing *P. aeruginosa* strains indicated that the formation of extracellular β -galactosidase correlated with the formation of large amounts of extracellular DNA (Allesen-Holm *et al.*, 2006).

P. aeruginosa colonizes the lungs of cystic fibrosis (CF) patients and is a major cause of lung deterioration, health decline and death of these patients (Høiby *et al.*, 2001). Several studies indicate that *P. aeruginosa* forms biofilms in the CF lung (e.g. Lam *et al.*, 1980; Baltimore *et al.*, 1989; Worlitzsch *et al.*, 2002; Høiby *et al.*, 2001), and the biofilm mode of growth is considered the major reason that these bacteria can not be eradicated by host defences or antibiotic treatment (Costerton *et al.*, 1999; Høiby *et al.*, 2001). The conditions that infecting bacteria encounter in the CF lung is of great interest, and artificial CF sputum media have been developed to enable experiments to be carried out under relevant and controllable conditions (Sriramulu *et al.*, 2005; Palmer *et al.*, 2005). *P. aeruginosa* cells in the CF lung are believed to be in intense competition for iron with the host (Haas *et al.*, 1991; Ratledge & Dover, 2000). Low iron concentrations were shown to promote biofilm formation in artificial CF sputum medium (Sriramulu *et al.*, 2005). In addition to low iron levels, the presence of extracellular DNA was shown to be important for *P. aeruginosa* biofilm formation in artificial sputum medium (Sriramulu *et al.*, 2005).

Knowledge about the factors that affect DNA release from *P. aeruginosa* is important for understanding the environmental cues that can lead to biofilm formation and increased pathogenicity of this organism. In this paper we present evidence that the level of iron in the medium affects *P. aeruginosa* DNA release and biofilm formation. The iron level was shown to have an effect on the amount of biofilm formed in the wells of microtitre trays, as well as on structural biofilm development in flow chambers, and on the development of subpopulations with increased tolerance toward antimicrobial compounds. Evidence is presented that iron affects *P. aeruginosa* DNA release and biofilm development by affecting the PQS signalling pathway.

METHODS

Bacteria and growth conditions. *P. aeruginosa* PAO1 was obtained from the Genetic Stock Center (strain PAO0001). The *lasI* *rhlI* derivative was constructed by allelic displacement in PAO1 as described by Hentzer *et al.* (2003). The *pqsA*, *pqsR* and *pqsL* mutants were constructed by D'Argenio *et al.* (2002) via transposon insertion in PAO1. The *gfp*-tagged PAO1 strain was constructed by insertion of a mini-Tn7-eGFP-Gm^r cassette as described by Klausen *et al.* (2003b). *Escherichia coli* strains MT102 and MC1000 were used for standard DNA manipulations. LB medium (Bertani, 1951) was used to cultivate *E. coli* strains. Batch cultivation of *P. aeruginosa* was carried out at 30 °C in AB minimal medium (Clark & Maaløe, 1967) supplemented with 30 mg glucose l⁻¹. *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. FeCl₃ was added to the media to give the final ferric iron concentrations described in the text. Selective media were supplemented with ampicillin (Ap; 100 mg l⁻¹), gentamicin (Gm; 60 mg l⁻¹), kanamycin (Km; 100 mg l⁻¹), streptomycin (Sm; 100 mg l⁻¹), or tetracycline (Tc; 20 mg l⁻¹). AB medium supplemented with 0.05 mg propidium iodide ml⁻¹ was used in the microtitre trays for quantification of extracellular DNA. DDAO [7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one)] was used at a concentration of 1 µM to stain extracellular DNA in flow-chamber-grown biofilms. Propidium iodide was used at a concentration of 0.01 mg ml⁻¹ to stain dead cells in flow-chamber-grown biofilms. Syto62 was used at a concentration of 1 µM to stain bacteria in flow-chamber-grown biofilms.

Measurements of *pvdD* expression. A classical β -galactosidase assay, described by Miller (1972), was used to measure expression of the *pvdD-lacZ* fusion in *P. aeruginosa* PAO1 cells transformed with the low-copy-number plasmid pPM190::*pvdD* (Rombel *et al.*, 1995), which carries the *pvdD* promoter fused to the *E. coli lacZ* gene.

Construction of the *pqsA::gfp(ASV)* reporter. The promoter-probe plasmid pAC37, containing a *P_{pqsA}::gfp(ASV)* transcriptional fusion, was constructed using standard techniques for DNA manipulations (Sambrook *et al.*, 1989). A *P. aeruginosa* DNA region containing the *P_{pqsA}* promoter was PCR amplified by use of the primers PA0996F (5'-GGAATTCTGATCAATGCCGTCGCCCCCTTGA-3') and PA0996R (5'-GGGTACCCATGACAGAACGTTCCCTCTT-3'). A *P. aeruginosa* DNA region containing the *P_{rhlR}* promoter and the *rhlR* gene was PCR amplified by use of the primers PA3477F (5'-CGTGATCAAACGGCTGGTCCGGCTCG-3') and PA3477R (5'-GGAATTCAAAAAGGCCATCCGTCAGGATGGCCTTCTAGATTACTACTCAGATGAGACCCAGCGCC-3'). An *E. coli* T2 transcriptional terminator and a unique *XbaI* cloning site were introduced in the PCR fragment through use of the PA3477R primer set. pJBA100, consisting of a *NotI* fragment with the *gfp(ASV)* gene from pJBA89

(Andersen *et al.*, 2001) cloned in the shuttle vector pUCP22*NotI*, was used as scaffold. pJBA100 was digested with *KpnI* and *EcoRI*; the PCR product containing the *P_{pgsA}* promoter was digested with *BclI* and *KpnI*; and the PCR product containing the *rhlR* gene was digested with *BclI* and *EcoRI*. The expression monitor pAC36 was constructed by ligating the three DNA fragments in one reaction. Plasmid pAC37 was constructed by deleting the *rhlR* gene on pAC36, via digestion with *XbaI* and *BclI*, Klenow treatment, and religation. The pAC37 plasmid was electroporated into *P. aeruginosa* PAO1 and the *P. aeruginosa lasI rhlI* mutant.

Measurement of biofilm and extracellular DNA in microtitre trays. The microtitre tray biofilm formation assay was performed essentially as described by O'Toole & Kolter (1998). Overnight cultures were grown in AB medium and diluted to OD₆₀₀ 0.001 with fresh AB medium. The diluted cultures were transferred to the wells of polystyrene microtitre trays (150 µl per well) and incubated for 24 h at 30 °C. The medium was removed from the wells and they were washed twice with 0.9 % NaCl, stained with 0.1 % crystal violet and washed twice with 0.9 % NaCl; the crystal violet-stained biofilms were then resuspended in 96 % ethanol, and biofilm cell-associated dye was measured as A₅₉₀. Measurements of extracellular DNA in *P. aeruginosa* microtitre tray cultures was done as described by Allesen-Holm *et al.* (2006). Microtitre tray cultures were started by diluting *P. aeruginosa* overnight cultures to OD₆₀₀ 0.001 in AB-glucose medium supplemented with 0.05 mM propidium iodide and FeCl₃ (as indicated) in wells of polystyrene microtitre plates (150 µl per well). The cultures were incubated for 24 h at 37 °C, after which propidium iodide absorbance was measured at 480 nm and cell density was measured at 600 nm by the use of a Wallac microplate reader.

DNase I treatment of biofilms in microtitre trays. Twenty-four-hour-old microtitre-tray-grown biofilms of PAO1 were washed twice with 0.9 % NaCl and treated with 0.9 % NaCl containing 100 µg DNase I ml⁻¹ for 3 h, after which the amount of biofilm was measured as described above.

Cultivation of biofilms in flow-chambers. Biofilms were grown in flow-chambers with individual channel dimensions of 1×4×40 mm. The flow system was assembled and prepared as described previously (Sternberg & Tolker-Nielsen, 2005). The flow-chambers were inoculated by injecting 350 µl overnight culture diluted to an OD₆₀₀ of 0.001 into each flow channel using a small syringe. After inoculation, the flow channels were left without flow for 1 h, after which medium flow was started using a Watson Marlow 205S peristaltic pump. The mean flow velocity in the flow-chambers was 0.2 mm s⁻¹, corresponding to laminar flow with a Reynolds number of 0.02.

Microscopy and image acquisition. All microscopy observations and image acquisitions were done with a Zeiss LSM510 confocal laser scanning microscope (CLSM) equipped with detectors and filter sets for monitoring of Gfp, propidium iodide Syto62 and DDAO fluorescence. Images were obtained using a 63×/1.4 objective or a 40×/1.3 objective. Simulated three-dimensional images and sections were generated using the IMARIS software package (Bitplane AG).

Assessment of extracellular DNA in flow-chamber-grown biofilms. DDAO was added to the medium to a final concentration of 1 µM to fluorescently stain extracellular DNA in flow-chamber-grown biofilms. DDAO fluorescence in different biofilms was recorded with fixed settings of the CLSM.

Antimicrobial treatment. Biofilm tolerance to EDTA or ciprofloxacin was assessed by irrigating 4-day-old flow-chamber-grown *P. aeruginosa* biofilms with medium containing 50 mM EDTA or 50 µg ciprofloxacin ml⁻¹ for 24 h, followed by staining of the dead cells

with propidium iodide, and CLSM image acquisition. To quantify survival rates, the bacteria from flow-chamber biofilms were recovered after EDTA treatment, ciprofloxacin treatment, or no antimicrobial treatment, by pumping bead-containing (Sigma glass-beads 212–300 µm) medium rapidly in and out of the flow-chambers using syringes. The bead-containing bacterial suspensions were vortex mixed, and the bacteria were plated on LB plates; c.f.u. were determined and survival rates calculated.

COMSTAT image analysis. CLSM images were analysed by use of the computer program COMSTAT (Heydorn *et al.*, 2000). A fixed threshold value and connected volume filtration were used for all image stacks.

RESULTS

We initially performed experiments to determine the iron status of *P. aeruginosa* in our AB-glucose medium supplemented with different concentrations of iron. Expression of the *pvdD* gene in *P. aeruginosa* is known to be suppressed by high levels of iron (Ochsner *et al.*, 2002). We measured β-galactosidase activity in microtitre tray cultures of *P. aeruginosa* harbouring the low-copy-number plasmid pPM190::pvdD, which carries the *pvdD* promoter fused to the *E. coli lacZ* gene (Rombel *et al.*, 1995). The β-galactosidase activity (Miller units) was 7757, 4944, 2773 and 2206 (three replicates, SD 581, 458, 347 and 58) in cultures supplemented with 5, 10, 50 and 100 µM FeCl₃. Accordingly, the selected range of iron concentrations in our medium evidently affected the iron status of the *P. aeruginosa* cells.

When *P. aeruginosa* PAO1 was cultivated in microtitre trays, a high cell density was reached in wells containing AB-glucose medium supplemented with 100 or 50 µM iron, whereas a slightly lower cell density was reached in wells containing AB-glucose medium supplemented with 10 µM iron, and a significantly lower cell density was reached in wells containing AB-glucose medium supplemented with 5 µM iron (Fig. 1A). However, the lowest iron concentration used did not limit growth dramatically, and the highest iron concentration used did not inhibit growth.

Iron affects *P. aeruginosa* DNA release and biofilm formation in microtitre trays

High concentrations of iron salts were reported to inhibit *P. aeruginosa* biofilm formation (Musk *et al.*, 2005). Because extracellular DNA is a major matrix component of *P. aeruginosa* biofilms (Whitchurch *et al.*, 2002; Nemoto *et al.*, 2003; Matsukawa & Greenberg, 2004; Allesen-Holm *et al.*, 2006), we found it of interest to investigate whether the presence of high levels of iron might inhibit the generation of extracellular DNA from *P. aeruginosa*. In accordance with the work of Musk *et al.* (2005), we found that biofilm formation by *P. aeruginosa* PAO1 in microtitre trays decreased with increasing iron concentrations (Fig. 1B). In order to quantify the extracellular DNA generated in the microtitre tray cultures (from planktonic as well as from biofilm cells), we supplemented the medium with propidium iodide (which fluoresces when it is bound to DNA,

Fig. 1. *P. aeruginosa* PAO1 was grown for 24 h in microtitre tray wells containing AB-glucose medium with different concentrations of FeCl_3 , then culture densities, biofilm amounts and extracellular DNA amounts were determined. (A) Final OD₆₀₀ of the cultures. (B) Relative amounts of biofilm in the wells determined via a crystal violet staining assay. (C) Relative amounts of extracellular DNA in the cultures determined via a propidium iodide staining assay. (D) Relative amounts of biofilm in the wells after 3 h of DNase I treatment. Means and standard deviations of eight replicates are shown.

and does not penetrate live bacteria) and measured the level of fluorescence in the cultures after 1 day of growth. The amount of extracellular DNA in *P. aeruginosa* PAO1 microtitre tray cultures was high in medium supplemented with 5 μM iron, and decreased with increasing iron concentrations (Fig. 1C). Treatment of 1-day-old *P. aeruginosa* PAO1 biofilms with DNase I led to dispersal of large amounts of the biofilms (Fig. 1D), showing that extracellular DNA plays a critical role in biofilm formation in the experimental setup used.

Iron affects the *Pseudomonas* quinolone signal (PQS) pathway

In a previous report, we presented evidence that release of extracellular DNA from *P. aeruginosa* populations was regulated through the *pqs* quorum-sensing system (Allesen-Holm *et al.*, 2006). To investigate whether the observed effect of iron on DNA release from *P. aeruginosa* populations occurs through the PQS system, we initially tested DNA release from different PQS mutants in microtitre tray cultures supplemented with different concentrations of iron. The mutants *pqsA* and *pqsR* were found to release less extracellular DNA than the wild-type in an

iron-independent manner, whereas a *pqsL* mutant was found to release more extracellular DNA than the wild-type via a pathway that was suppressed by high iron concentrations (Fig. 2). These results are in agreement with evidence reported by D'Argenio *et al.* (2002) that the *pqsA* and *pqsR* mutants were deficient in PQS production and autolysis, whereas the *pqsL* mutant overproduced PQS and was hyper-autolysing.

To further investigate whether the observed effect of iron on DNA release and biofilm formation might be caused by an effect on the PQS pathway, we constructed a plasmid-based *pqsA::gfp(ASV)* transcriptional reporter (as detailed in Methods). The *gfp(ASV)* gene encodes an unstable version of Gfp (Andersen *et al.*, 1998) and the *pqsA::gfp(ASV)* reporter-construct is thus useful for monitoring *pqsABCDE* expression. The plasmid with the reporter-construct was transferred to the *P. aeruginosa* wild-type as well as to an isogenic *P. aeruginosa lasI rhII* quorum-sensing mutant, and *pqsA::gfp(ASV)* expression was measured in microtitre tray cultures. The experiments confirmed that the *pqsABCDE* operon is under *N*-acylhomoserine lactone (AHL) quorum-sensing control, as expression of the *pqsA::gfp(ASV)* reporter consistently was lower in the *lasI rhII* quorum-sensing mutant

Fig. 2. Relative amounts of extracellular DNA, determined via a propidium iodide staining assay, in microtitre tray cultures of PAO1 wild-type (black bars), *pqsL* (dark grey bars), *pqsA* (light grey bars) and *pqsR* (white bars) grown in AB-glucose medium containing different amounts of FeCl₃. Means and standard deviations of eight replicates are shown.

than in the wild-type (Fig. 3A). Transcription of the *pqsABCDE* operon in the *P. aeruginosa* wild-type was found to be high in medium supplemented with 5 μM iron, and decreased with increasing iron concentrations (Fig. 3A). In the *lasI rhII* quorum-sensing mutant there was also an inverse relation between *pqsA::gfp(ASV)* expression and the level of iron in the medium (Fig. 3A), suggesting that the effect of iron on *pqsABCDE* expression to some extent is independent of the AHL quorum-sensing system. DNA release in the microtitre tray cultures was found to correlate with the expression of the *pqsABCDE* operon (Fig. 3B). Based on these results, we suggest that iron affects *P. aeruginosa* DNA release and biofilm formation by affecting the PQS signalling pathway.

In order to address whether the observed effects of ferric iron supplementation could be due to induction of an oxidative stress response in the *P. aeruginosa* cells, we investigated microtitre tray cultures supplemented with different concentrations (ranging from 0 to 10 mM) of H₂O₂, and found no reduction in *pqsA::gfp(ASV)* expression, DNA release, or biofilm formation (data not shown), indicating that the observed effects of iron are not due to an oxidative stress response.

Iron affects *P. aeruginosa* DNA release and structural biofilm development in flow-chambers

In order to examine the effect of iron concentration on DNA release in *P. aeruginosa* biofilms, we grew the PAO1 wild-type in flow-chambers irrigated with normal FAB medium (containing 1 μM iron) or FAB100 medium (containing 100 μM iron). Because of the continuous supply of medium, the amount of iron available to the bacteria will be higher in the flow-chamber setup than in the microtitre tray setup, and we therefore chose to use FAB, containing 1 μM iron, as the medium with the lowest iron concentration in the investigations. After 4 days of growth, the extracellular DNA in the biofilms was visualized by staining with the compound DDAO followed by CLSM. In agreement with our previous study (Allesen-Holm *et al.*, 2006), the biofilms grown in FAB medium contained large amounts of extracellular DNA in the part of the biofilm close to the substratum and in the outer region of the stalk portions of the mushroom-shaped multicellular structures (Fig. 4A). In agreement with the quantifications of extracellular DNA and biofilm formation in the microtitre tray assays (described above), the biofilms grown in FAB100 medium contained less extracellular DNA and less biomass than the biofilms grown in normal FAB medium (Fig. 4B). In addition, biofilms grown in FAB100 medium appeared less spatially

Fig. 3. (A) Expression of a plasmid-borne *pqsA::gfp(ASV)* fusion in microtitre tray cultures of the PAO1 wild-type (black bars) and a *lasI rhII* mutant (grey bars) grown in AB-glucose medium with different concentrations of FeCl₃. (B) Relative amounts of extracellular DNA, determined via a propidium iodide staining assay, in microtitre tray cultures of the PAO1 wild-type (black bars) and *lasI rhII* mutant (grey bars) grown in AB-glucose medium containing different amounts of FeCl₃. Means and standard deviations of eight replicates are shown.

Fig. 4. Four-day-old biofilms of *gfp*-tagged PAO1 grown in FAB medium (A) or FAB100 medium (B) were stained with DDAO and images visualizing cells (green) and extracellular DNA (appearing red or yellow) were acquired by CLSM. The central pictures show horizontal optical sections, and the flanking pictures show vertical optical sections. Bars, 20 μm .

organized and did not contain the mushroom-shaped multicellular structures typical of the biofilms grown in normal FAB medium (Fig. 4). The difference in biomass and spatial structure between biofilms grown in normal FAB medium and FAB100 medium was confirmed by COMSTAT analysis (Table 1).

Iron affects expression of the *pqsABCDE* operon in flow-chamber-grown *P. aeruginosa* biofilms

In order to investigate expression of the *pqsABCDE* operon in *P. aeruginosa* biofilms, we grew PAO1 containing the *pqsA::gfp(ASV)* reporter plasmid in flow-chambers irrigated with FAB medium or FAB100 medium. In FAB medium, expression of the *pqsA::gfp(ASV)* fusion was detectable at day 1 (Fig. 5A) and reached the highest expression level at day 2 where it was localized mainly in the outer part of the microcolonies (Fig. 5C). After day 2, expression of the *pqsA::gfp(ASV)* fusion decreased, and at day 4 only a weak green signal could be detected in the stalk portion of the mushroom-shaped multicellular structures (Fig. 5E). In FAB100 medium, there was significantly less expression of the *pqsA::gfp(ASV)* fusion, and this expression appeared less coordinated in time and space (Fig. 5B, D and F).

Iron affects tolerance to antimicrobial compounds in flow-chamber-grown *P. aeruginosa* biofilms

Since we had found that the level of iron in the medium may control whether a structured or an unstructured *P. aeruginosa* biofilm is formed, and because tolerance to antimicrobial compounds in biofilms may be related to the spatial structure, we found it of interest to investigate tolerance to the antibiotic ciprofloxacin and to the antimicrobial compound EDTA in *P. aeruginosa* biofilms grown in FAB medium or FAB100 medium. After 4 days of growth, the biofilms were treated with either ciprofloxacin or EDTA, after which the dead bacteria in the biofilms were visualized by staining with propidium iodide. In 4-day-old *P. aeruginosa* PAO1 biofilms grown in FAB medium, ciprofloxacin treatment caused killing of the bacteria located in the outer part of the cap portion of the mushroom-shaped structures (Fig. 6C), whereas EDTA treatment caused killing of the cells located within the mushroom-shaped structures (Fig. 6E). In 4-day-old *P. aeruginosa* PAO1 biofilms grown in FAB100 medium, treatment with ciprofloxacin or EDTA appeared to kill a large proportion of the bacteria, and there was no special area for the ciprofloxacin- or EDTA-mediated killing (Fig. 6D, F). Propidium iodide staining of biofilms that had not been treated with antibiotic showed that virtually no dead cells

Table 1. Quantification of biofilm architecture

The results are means of datasets obtained from analysis of eight CLSM images acquired at random positions in each of the biofilms. Standard deviations are shown in parentheses.

Biofilm medium	Total biomass ($\mu\text{m}^3 \mu\text{m}^{-2}$)	Mean thickness (μm)	Roughness coefficient	Surface area covered (μm^2)	Surface/ volume ($\mu\text{m}^2 \mu\text{m}^{-3}$)	Max. thickness (μm)
FAB (containing 1 μM FeCl_3)	40.06 (1.97)	42.82 (2.74)	0.39 (0.08)	8.71×10^5 (2.72×10^5)	0.41 (0.12)	96.63 (3.42)
FAB100 (containing 100 μM FeCl_3)	13.08 (4.76)	17.56 (5.41)	0.59 (0.18)	1.21×10^6 (3.30×10^5)	1.81 (0.36)	59.75 (2.12)

Fig. 5. Biofilms of PAO1 containing a *pqsA-gfp(ASV)* reporter were grown for 1 (A, B), 2 (C, D) and 4 (E, F) days in flow chambers irrigated with FAB medium (A, C, E) or FAB100 medium (B, D, F). The cells were stained with Syto62, and images visualizing cells (red) and *pqsA-gfp(ASV)* expression (appearing yellow) were acquired by CLSM. The central pictures show horizontal optical sections, and the flanking pictures show vertical optical sections. Bars, 20 μ m.

were present in the biofilms before antibiotic treatment, and that the propidium iodide staining did not visualize extracellular DNA with the setting of the CLSM that was used (Fig. 6A, B). (Propidium iodide does not give as high a signal as DDAO, and with normal settings of the CLSM it will therefore mainly visualize dead cells where the concentration of DNA is high compared to in the biofilm matrix.)

Assessment of survival in the biofilms through recovery of the bacteria from the flow-chambers, after EDTA treatment, ciprofloxacin treatment, or no antimicrobial treatment, followed by plating and c.f.u. counting, confirmed that the structured *P. aeruginosa* biofilms grown in FAB medium displayed higher tolerance to the ciprofloxacin and EDTA treatment than the unstructured *P. aeruginosa* biofilms

grown in FAB100 medium. C.f.u. from ciprofloxacin-treated biofilms divided by c.f.u. from untreated biofilms was 0.22 (three replicates, SD 0.13) for the biofilms grown in FAB, and 0.02 (three replicates, SD 0.001) for the biofilms grown in FAB100. C.f.u. from EDTA-treated biofilms divided by c.f.u. from untreated biofilms was 0.55 (three replicates, SD 0.095) for the biofilms grown in FAB and 0.21 (three replicates, SD 0.082) for the biofilms grown in FAB100.

DISCUSSION

A number of recent reports suggest that iron affects biofilm formation by *P. aeruginosa*. Experiments using iron-chelating lactoferrin suggested that a minimum concentration of free

Fig. 6. Four-day-old biofilms of *gfp*-tagged PAO1 grown in FAB medium (A, C, E) or FAB100 medium (B, D, F) were either not treated (A, B), treated with ciprofloxacin (C, D), or treated with EDTA (E, F), after which they were stained with propidium iodide and images were acquired by CLSM. Live cells appear green and dead cells appear yellow or red. The central pictures show horizontal optical sections, and the flanking pictures show vertical optical sections. Bars, 20 μ m.

iron is necessary for the formation of structured *P. aeruginosa* biofilms in a flow-chamber system (Singh *et al.*, 2002; Banin *et al.*, 2005). On the other hand, high concentrations of iron were shown to suppress *P. aeruginosa* biofilm formation in both microtitre tray and flow-chamber systems (Musk *et al.*, 2005), and low iron concentrations were shown to be required for *P. aeruginosa* biofilm (microcolony) formation in artificial sputum medium (Sriramulu *et al.*, 2005). In agreement with these previous studies, we found in the present study that in our microtitre tray and flow-chamber setups low iron levels promoted biofilm formation while high iron levels suppressed biofilm formation. Experiments involving DNase I treatment of

biofilms in microtitre trays (this study) or flow-chambers (Allesen-Holm *et al.*, 2006) suggested that extracellular DNA is an important constituent of the biofilm matrix. Furthermore, evidence was presented that the formation of extracellular DNA in *P. aeruginosa* biofilms is regulated via the PQS quorum-sensing system (Allesen-Holm *et al.*, 2006). In this paper, we present evidence that low iron concentrations promote *P. aeruginosa* biofilm development via up-regulation of the *pqs* genes and the formation of extracellular DNA, and that high levels of iron suppress *P. aeruginosa* biofilm development via down-regulation of the *pqs* genes and the formation of extracellular DNA. In agreement with our present and previous work, a study

comparing different microarray results identified the PQS regulator gene *pqsR* as being among genes that are induced at low iron levels and also positively controlled by quorum sensing (Cornelis & Aendekerk, 2004).

Under specific conditions in flow-chamber systems (e.g. the glucose FAB medium used in the present study), *P. aeruginosa* forms heterogeneous biofilms with mushroom-shaped multicellular structures via a process that involves formation of microcolonies in the initial phase and subsequent formation of cap-shaped structures on top of the microcolonies in the later phase (e.g. Klausen *et al.*, 2003a). In agreement with the present study, the extracellular DNA in such *P. aeruginosa* biofilms was shown to be present in high concentrations close to the substratum and in the outer part of the microcolonies in the initial phase of biofilm development, and in the outer region of the stalk portion of the mushroom-shaped multicellular structures in the later phase of biofilm development (Allesen-Holm *et al.*, 2006). In the present study we observed that when *P. aeruginosa* formed biofilms in flow-chambers irrigated with FAB medium, expression of a *pqsA::gfp(ASV)* fusion was detectable in 1-day-old biofilms and reached the highest expression level at day 2, where it was localized mainly in the outer part of the microcolonies. After day 2, expression of the *pqsA::gfp(ASV)* fusion in the biofilms decreased, and at day 4 only a weak green signal could be detected in the stalk portion of the mushroom-shaped multicellular structures. This spatio-temporal expression pattern of *pqsABCDE* is in accordance with the location of extracellular DNA in *P. aeruginosa* biofilms reported here and by Allesen-Holm *et al.* (2006). In the medium containing a high iron concentration, there was significantly less expression of the *pqsA::gfp(ASV)* fusion, and this expression appeared less coordinated in time and space. In agreement with the quantifications of extracellular DNA and biofilm formation in the microtitre tray assays, and in agreement with the role of the *pqs* genes in DNA release (Allesen-Holm *et al.*, 2006), the biofilms grown at high iron concentration contained less extracellular DNA and less biomass than the biofilms grown in normal FAB medium. In addition, biofilms grown at high iron concentration appeared less spatially organized and did not develop mushroom-shaped multicellular structures.

The cap-forming and the stalk-forming subpopulation of the mushroom-shaped multicellular structures in *P. aeruginosa* biofilms in many cases display differential tolerance to antimicrobial compounds. For example, the antibiotic tobramycin was shown to kill preferentially bacteria located in the outer part of the cap portion of the mushroom-shaped structures, whereas the antibiotic colistin, the detergent SDS and the chelator EDTA were shown to kill preferentially bacteria in the stalk portion of the mushroom-shaped structures (Bjarnsholt *et al.*, 2005; Banin *et al.*, 2006; Haagensen *et al.*, 2007). Since we had found that the level of iron in the medium may control whether a structured or an unstructured *P. aeruginosa*

biofilm is formed, we found it of interest to investigate tolerance to antimicrobial compounds in *P. aeruginosa* biofilms grown in FAB medium or in FAB100 high-iron medium. Ciprofloxacin treatment and EDTA treatment caused killing of cells located in the cap portion and stalk portion, respectively, of the mushroom-shaped structures of 4-day-old *P. aeruginosa* biofilms grown in FAB medium, whereas there was no special area for ciprofloxacin- or EDTA-mediated killing in 4-day-old biofilms grown in high-iron medium. In addition, the structured *P. aeruginosa* biofilms, grown in FAB medium, displayed higher tolerance to the antimicrobial treatment than the unstructured *P. aeruginosa* biofilms, grown in high-iron medium.

The formation of *P. aeruginosa* extracellular DNA is evidently regulated by both quorum sensing and the level of available iron. Other processes that appear to be subject to regulation by the same cues include elastase production and Mn-superoxide dismutase (SOD) production, which were both shown to be quorum-sensing controlled (Hassett *et al.*, 1999; Winson *et al.*, 1995), and also regulated by iron so that they were induced under low-iron conditions but repressed under high-iron conditions (Bjorn *et al.*, 1979; Hassett *et al.*, 1997; Sokol *et al.*, 1982; Bollinger *et al.*, 2001). The fact that iron regulation of these processes to some extent is independent of quorum-sensing regulation makes nutritional override of quorum sensing possible. Other examples of possible nutritional override of quorum sensing include pyocyanin production, which is positively controlled by quorum sensing (Brint & Ohman, 1995), but can also be induced by phosphate starvation (Hassett *et al.*, 1992). The possibility of nutritional override of quorum sensing should be taken into account when possible strategies for manipulating quorum sensing for controlling bacterial infections are considered.

Evidence was recently presented that biofilm formation by *Staphylococcus aureus* is induced at low iron concentrations and repressed at high iron concentrations via a mechanism that does not affect expression of polymeric *N*-acetylglucosamine, which is the major exopolysaccharide implicated in *S. aureus* biofilm formation (Johnson *et al.*, 2005). Our unpublished results suggest that extracellular DNA plays a role in *S. aureus* biofilm formation, and that formation of *S. aureus* extracellular DNA occurs at high levels in low-iron medium and at low levels in high-iron medium. It is possible, therefore, that there is a connection between iron concentration, extracellular DNA and biofilm formation also for the Gram-positive organism *S. aureus*.

The finding that high amounts of iron may inhibit *P. aeruginosa* biofilm formation raises the question whether iron might be used to treat biofilm infections in medical settings. However, because the lack of free iron in the human body in most cases is limiting bacterial growth (Weinberg, 1999), treatment of biofilm-based infections with iron might not be feasible. Nevertheless, the possibility exists that a combination treatment involving antibiotics and iron might be useful. The finding that iron can suppress biofilm

formation may motivate a search for similar compounds that can suppress biofilm formation but do not stimulate bacterial growth.

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