Effects of iron on DNA release and biofilm development by \textit{Pseudomonas aeruginosa}

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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 \textit{et al}, 2002; Klausen \textit{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 \textit{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 \textit{et al}, 2002).

Evidence for a role of extracellular DNA as matrix component in \textit{Pseudomonas aeruginosa} biofilms has been presented both for the \textit{P. aeruginosa} PAO1 reference strain and for clinical \textit{P. aeruginosa} isolates (Whitchurch \textit{et al}, 2002; Nemoto \textit{et al}, 2003). \textit{P. aeruginosa} PAO1 biofilm development in the wells of microtitre plates was attenuated by the presence of DNase I, and biofilm formation by \textit{P. aeruginosa} PAO1 in flow-chambers was almost absent when the flow-chambers were irrigated with medium containing DNase I (Whitchurch \textit{et al}, 2002). In addition, young \textit{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 \textit{P. aeruginosa} PAO1 biofilms were not dispersed by DNase I treatment, suggesting that components other than extracellular DNA stabilize mature \textit{P. aeruginosa} PAO1 biofilms (Whitchurch \textit{et al}, 2002). Matsukawa & Greenberg (2004) investigated the composition of the extracellular matrix of mature \textit{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 \textit{P. aeruginosa} PAO1 biofilms, Nemoto

Abbreviations: AHL, N-acylhomoserine lactone; CLSM, confocal laser scanning microscope/microscopy; DDAO, 7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one); PQS, \textit{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, phtAB and pqsH genes, as well as on a LysR-type regulator encoded by the pqsR gene (also known as myfR) (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 (Hoiby 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; Hoiby 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; Hoiby 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 PA00001). The las rhl derivative was constructed by allelic displacement in PA01 as described by Hentze et al. (2003). The pqsA, pqsR and pqsL mutants were constructed by D’Argenio et al. (2002) via transposon insertion in PA01. The gfp-tagged PA01 strain was constructed by insertion of a mini-Tn7-eGFP-Gm' 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 & Maaloe, 1967) supplemented with 30 mg glucose 1⁻¹. 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 PA01 cells transformed with the low-copy-number plasmid pMP190::pvdD (Rombol et al., 1993), 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’-GGATTCCTCTGATACATGCGTGGCCCTCTGGAG-3’) and PA0996R (5’-GGTACCATATTCGACGAACTTGGCTC-3’). A P. aeruginosa DNA region containing the P_rhl promoter and the rhlR gene was PCR amplified by use of the primers PA3477F (5’-CGTGATCAACGGCGTGGTCCCCGTTG-3’) and PA3477R (5’-GGATTCGAAAGCGCCATCCGCTAGGCTGTCCTAGATTTCTTACTCATGAGATGAGGCGCC-3’). An E. coli T2 transcriptional terminator and a unique XbaI cloning site were introduced in the PCR fragment through use of the PA3477 primer set. pJBA100, consisting of a NotI fragment with the gfp(ASV) gene from pJBA89.
(Andersen et al., 2001) cloned in the shuttle vector pUCP22NotI, was used as scaffold. pBIA100 was digested with KpnI and EcoRI; the PCR product containing the P_{paP} promoter was digested with BclI and KpnI; and the PCR product containing the rhIR 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 rhIR gene on pAC36, via digestion with XhoI 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 OD_{600} 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 A_{590}. 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 OD_{600} 0.001 in AB-glucose medium supplemented with 0.05 mM propidium iodide and FeCl_3 (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 1x4x 40 mm. The flow system was assembled and prepared as described previously (Sternberg & Tolkier-Nielsen, 2005). The flow-chambers were inoculated by injecting 350 μl overnight culture diluted to an OD_{600} 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 63x/1.4 objective or a 40x/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 determined and survival rates calculated.

**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 212–200 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; cf.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 harbouing the low-copy-number plasmid pPM190::pvdD, which carries the pvdD promoter fused to the E. coli lacZ gene (Rombol 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 FeCl_3. 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 Mus 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,
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 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.

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 rhlI* 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 rhlI* quorum-sensing mutant.
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 mM iron, and decreased with increasing iron concentrations (Fig. 3A). In the lasI rhlI 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. 2. Relative amounts of extracellular DNA, determined via a propidium iodide staining assay, in microtitre tray cultures of the 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.](image)

![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 rhlI 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 rhlI mutant (grey bars) grown in AB-glucose medium containing different amounts of FeCl3. Means and standard deviations of eight replicates are shown.](image)
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 \textit{pqsABCDE} operon in flow-chamber-grown \textit{P. aeruginosa} biofilms

In order to investigate expression of the \textit{pqsABCDE} operon in \textit{P. aeruginosa} biofilms, we grew PAO1 containing the \textit{pqsA::gfp(ASV)} reporter plasmid in flow-chambers irrigated with FAB medium or FAB100 medium. In FAB medium, expression of the \textit{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 \textit{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 \textit{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 \textit{P. aeruginosa} biofilms

Since we had found that the level of iron in the medium may control whether a structured or an unstructured \textit{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 \textit{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 \textit{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 \textit{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.

<table>
<thead>
<tr>
<th>Biofilm medium</th>
<th>Total biomass ((\mu m^3/\mu m^2))</th>
<th>Mean thickness ((\mu m))</th>
<th>Roughness coefficient</th>
<th>Surface area covered ((\mu m^2))</th>
<th>Surface/volume ((\mu m^2/\mu m^3))</th>
<th>Max. thickness ((\mu m))</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAB (containing 1 (\mu M) FeCl(_3))</td>
<td>40.06 (1.97)</td>
<td>42.82 (2.74)</td>
<td>0.39 (0.08)</td>
<td>8.71(\times)10(^5) (2.72(\times)10(^5))</td>
<td>0.41 (0.12)</td>
<td>96.63 (3.42)</td>
</tr>
<tr>
<td>FAB100 (containing 100 (\mu M) FeCl(_3))</td>
<td>13.08 (4.76)</td>
<td>17.56 (5.41)</td>
<td>0.59 (0.18)</td>
<td>1.21(\times)10(^6) (3.30(\times)10(^5))</td>
<td>1.81 (0.36)</td>
<td>59.75 (2.12)</td>
</tr>
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Fig. 4. Four-day-old biofilms of \textit{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 \(\mu m\).
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 \textit{P. aeruginosa} biofilms grown in FAB medium displayed higher tolerance to the ciprofloxacin and EDTA treatment than the unstructured \textit{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 \textit{P. aeruginosa}. Experiments using iron-chelating lactoferrin suggested that a minimum concentration of free
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

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**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 μm.
comparing different microarray results identified the PQS regulator gene \textit{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), \textit{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 \textit{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 \textit{P. aeruginosa} formed biofilms in flow-chambers irrigated with FAB medium, expression of a \textit{pqsA}::\textit{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 \textit{pqsA}::\textit{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 \textit{pqsA}BCDE is in accordance with the location of extracellular DNA in \textit{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 \textit{pqsA}::\textit{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 \textit{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 \textit{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 \textit{P. aeruginosa} biofilm is formed, we found it of interest to investigate tolerance to antimicrobial compounds in \textit{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 \textit{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 \textit{P. aeruginosa} biofilms, grown in FAB medium, displayed higher tolerance to the antimicrobial treatment than the unstructured \textit{P. aeruginosa} biofilms, grown in high-iron medium.

The formation of \textit{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 \textit{Staphylococcus aureus} is induced at low iron concentrations and repressed at high iron concentrations via a mechanism that does not affect expression of polymeric \textit{N-acetylglucosamine}, which is the major exopolysaccharide implicated in \textit{S. aureus} biofilm formation (Johnson et al., 2005). Our unpublished results suggest that extracellular DNA plays a role in \textit{S. aureus} biofilm formation, and that formation of \textit{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 \textit{S. aureus}.

The finding that high amounts of iron may inhibit \textit{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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