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Gene Expression in *Pseudomonas aeruginosa*: Evidence of Iron Override Effects on Quorum Sensing and Biofilm-Specific Gene Regulation

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Prior studies established that the *Pseudomonas aeruginosa* oxidative stress response is influenced by iron availability, whereas more recent evidence demonstrated that it was also controlled by quorum sensing (QS) regulatory circuitry. In the present study, *sodA* (encoding manganese-cofactored superoxide dismutase [Mn-SOD]) and Mn-SOD were used as a reporter gene and endogenous reporter enzyme, respectively, to reexamine control mechanisms that govern the oxidative stress response and to better understand how QS and a nutrient stress response interact or overlap in this bacterium. In cells grown in Trypticase soy broth (TSB), Mn-SOD was found in wild-type stationary-phase planktonic cells but not in a *lasI* or *lasR* mutant. However, Mn-SOD activity was completely suppressed in the wild-type strain when TSB was supplemented with iron. Reporter gene studies indicated that *sodA* transcription could be variably induced in iron-starved cells of all three strains, depending on growth stage. Iron starvation induction of *sodA* was greatest in the wild-type strain and least in the *lasR* mutant and was maximal in stationary-phase cells. Reporter experiments in the wild-type strain showed increased *lasI*::*lacZ* transcription in response to iron limitation, whereas the expression level in the *las* mutants was minimal and iron starvation induction of *lasI*::*lacZ* did not occur. Studies comparing Mn-SOD activity in *P. aeruginosa* biofilms and planktonic cultures were also initiated. In wild-type biofilms, Mn-SOD was not detected until after 6 days, although in iron-limited wild-type biofilms Mn-SOD was detected within the initial 24 h of biofilm establishment and formation. Unlike planktonic bacteria, Mn-SOD was constitutive in the *lasI* and *lasR* mutant biofilms but could be suppressed if the growth medium was amended with 25 μM ferric chloride. This study demonstrated that (i) the nutritional status of the cell must be taken into account when one is evaluating QS-based gene expression; (ii) in the biofilm mode of growth, QS may also have negative regulatory functions; (iii) QS-based gene regulation models based on studies with planktonic cells must be modified in order to explain biofilm gene expression behavior; and (iv) gene expression in biofilms is dynamic.

Pseudomonas aeruginosa is ubiquitous, being found in diverse environments such as soil, freshwater, and marine environments. It is also an opportunistic pathogen of the airways of cystic fibrosis patients and in immunocompromised hosts including cancer, AIDS, and burn patients (16). Similar to other pathogens and gram-negative bacteria, *P. aeruginosa* has a global regulatory system known as quorum sensing (QS) that controls expression of numerous genes, many of which are associated with virulence (11, 37). Bacterial QS, or cell-to-cell communication, is a process in gram-negative and some gram-positive bacteria where low-molecular-weight diffusible molecules synthesized by one cell trigger gene activation in other cells (17). In gram-negative bacteria, the signaling molecules are either homoserine lactone/acyl side chain based (HSLs; autoinducers), diketopiperazine (29), or via 2-heptyl-3-hydroxy-4-quinolone (45), while gram-positive bacteria use small peptides. Because of its aforementioned ubiquity in nature and its importance in disease, *P. aeruginosa* is a model organism for QS study.

QS is viewed as a cell density-dependent phenomenon that allows bacteria to communicate, sense population density, and ultimately coordinate transcription of many genes. Bacteria monitor their population by sensing the level of autoinducer signal molecules (17). HSL-based QS in *P. aeruginosa* is a multitiered process governed by two gene tandems, *lasR-lasI* and *rhlR-rhlI* (41–43). The *las* system is composed of LasR, a transcriptional regulator protein, and LasI, an autoinducer synthase that produces one of the three known *Pseudomonas* HSLs, PAI-1 [*N*-(3-oxododecanoyl)-L-homoserine lactone]. The second tier consists of RhlR, which is also a transcriptional regulator, and RhlI, an autoinducer synthase that catalyzes the synthesis of a second HSL, PAI-2 (*N*-butyryl-L-homoserine lactone). PAI-1 interacts with the regulator LasR to activate transcription of target genes (42). The LasR–PAI-1 complex will activate the transcription of *lasI* and several genes important in defense against oxidative stress such as those coding for the major catalase, KatA, and the manganese-cofactored su-

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peroxide dismutase (Mn-SOD) (27). Further, recent work by Whiteley et al. has identified many other QS-regulated genes that were previously unrecognized (52).

Whether considered in either disease or environmental settings, an important aspect of *P. aeruginosa* ecology is its propensity to form biofilms. *P. aeruginosa* biofilms have high cell densities and an architecture that typically consists of highly ordered mushroom- and pillar-like structures (7). This important aspect of *P. aeruginosa* biology has also been shown to be influenced by QS (8). QS-deficient mutants form a thin, tightly packed biofilm, differing markedly from wild-type biofilm architecture, suggesting that particular aspects of biofilm cell physiology are under control of QS and are important for normal biofilm formation. The physiology of bacterial biofilms is viewed to be different from that of planktonic cultures (7), but the true extent of such potential differences is still poorly understood.

We are examining *P. aeruginosa* biofilm responses to environmental stimuli as a means of studying gene expression and physiology of biofilm bacteria. We have elected to focus on the oxidative stress response because our knowledge of the antioxidant responses in this organism is firmly grounded genetically and physiologically (20–24) and because antioxidant enzymes are of central importance to the pathogenicity of this organism (26). Further, we have recently found that key components of the oxidative stress response are regulated by QS (27). Curiously, earlier studies had also implicated iron availability as a significant controlling factor in expression levels of antioxidant enzymes in *P. aeruginosa* (20–27). As QS has thus far been found to exert its effects when cell densities are high, a condition which is found in biofilms and which can lead to localized areas of high nutrient demand, we have hypothesized that nutrient limitation may also be an important factor to consider in studies aimed at understanding QS and biofilm biology (28). The availability of well-defined QS mutants offers an excellent opportunity to examine and compare gene expression in biofilms and planktonic cells under conditions where the availability of a specific nutrient can be conveniently and reliably manipulated.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *P. aeruginosa* wild-type strain PAO1 (30), the *lasI*::Tn*10* mutant PAO-JP1 (44), and the *lasR* mutant PAO-R1 (42) were used in this study. Plasmids pDJH201 (contains *sodA*::*lacZ* [27]) and pPCS223 (contains *lasI*::*lacZ* [51]) were used in reporter gene experiments. These plasmids were introduced into the different strains by electroporation and maintained with carbenicillin (300 mg · liter^{-1}). In each case, plasmid transformation was verified by restriction enzyme analysis of plasmid preparations (47) of the different transformants. In some experiments, iron bioavailability in the medium was manipulated by the addition of iron (25 μM $FeCl_3$) or the iron-specific chelator 2,2-dipyridyl (500 μM for Trypticase soy broth [TSB] or 50 μM for 1/10 TSB).

Planktonic cultures were grown in TSB at 37°C in a rotary shaker at 300 rpm. Culture volumes did not exceed 10% of the flask volume to ensure maximum aeration. Biofilms were cultured using a drip flow reactor system previously described by Huang et al. (31) and included 316L stainless steel slides (1.3 by 7.6 cm) as the substratum. Briefly, 10 ml of 1/10 TSB was added to each chamber (four chambers per reactor), followed by inoculation with 1 ml of stationary-phase culture of the test strain grown in TSB. The reactor was then incubated horizontally at 37°C for 24 h to allow bacterial attachment to the substratum. Following the attachment period, the reactor was inclined 10° and a constant drip of 1/10 TSB was allowed to flow over the slides at a rate of 50 ml · h^{-1}. Biofilms were cultured in a 37°C incubator. To achieve this, the sterile 1/10 TSB contained in the external carboy was preequilibrated to 37°C prior to entry into the drip flow reactor. To accomplish this, the medium was first preheated to 42°C by pumping through masterflex silicone tubing coiled in a 42°C water bath fixed atop the incubator chamber. The feed tubing leaving the 42°C water bath was foam insulated (to reduce thermal loss) and channeled through the heat vent hole of the culture incubator. A mercury thermometer was attached to the medium flow tubing via aluminum tape, providing for isothermic association with the tubing and verifying that the temperature of medium was 37°C as it entered the incubator chamber. A final temperature equilibration step designed to guarantee appropriate temperature involved additional coiling (3-m flow length) of the feed tubing in distilled water (2-liter beaker) that was equilibrated at 37°C within the chamber. This ensured a final medium temperature of 37°C prior to entry into the drip flow reactor. Silicone tubing exiting each reactor chamber was used to pump the waste out of the chamber and into external waste carboys.

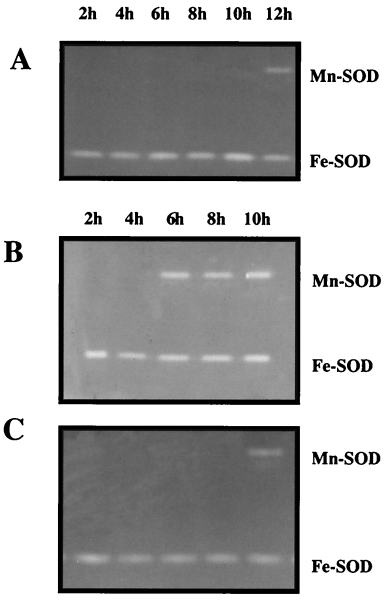

FIG. 1. SOD activity in planktonic cells cultured in TSB. Cell extracts (50 μg of protein per lane) of each strain were prepared at different time points corresponding to different growth stages, 6 h (mid-log phase) and 10 h (early stationary phase). SOD activity stains of wild-type strain PAO1 cultured in TSB (A), PAO1 in TSB amended with the iron-specific chelator 2,2-dipyridyl (B), and the *lasI* mutant PAO-JP1 in TSB amended with the iron-specific chelator 2,2-dipyridyl (C) were prepared as described in Materials and Methods. Results are of one of three independent experiments demonstrating this response.

Cell extract preparation, nondenaturing gel electrophoresis, and enzyme and reporter activity. Nondenaturing gel electrophoresis methods were as described by Hassett et al. (26). Briefly, cell extracts were prepared from cultures harvested by centrifugation at 10,000 × *g* for 10 min at 4°C. Bacteria were washed twice in ice-cold 50 mM potassium phosphate buffer (pH 7.0) and sonicated in an ice water bath for 30 s. The sonicate was clarified by centrifugation at 13,000 × *g* for 10 min at 4°C. Extract protein content was estimated by the Bradford assay (2). SOD activity stains in nondenaturing gels were used to semiquantatively follow induction of Mn-SOD and to assess its activity separately from the constitutively expressed Fe-SOD (coded by *sodB*) (26). Gel images were computer scanned and stored as Powerpoint image files. LacZ reporter enzyme expression was measured as β-galactosidase activity as previously described by Miller (38).

RESULTS

Mn-SOD regulation. Initial experiments compared Mn-SOD levels in the *P. aeruginosa* wild-type strain PAO1 and the *lasI* mutant PAO-JP1. In PAO1, Mn-SOD activity was detectable in stationary-phase cells (t = 12 h) (Fig. 1A), which correlates with the production and accumulation of the QS autoinducer molecule PAI-1 (27). Mn-SOD was absent from

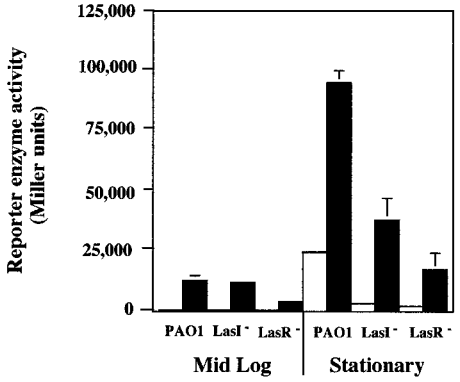

FIG. 2. Transcription of *sodA* in mid-log-phase and stationary-phase cells of PAO1, PAO-JP1, and PAO-R1 as affected by iron limitation. Relative transcription levels were determined by measuring the reporter enzyme β-galactosidase as described by Miller (38) in planktonic cells harvested at mid-log (6 h) and late stationary (16 h) phases. Open bars, cells grown in TSB; filled bars, cells grown in TSB containing the iron-specific chelator 2,2-dipyridyl. The data represent the mean of three independent experiments (two to three cultures per experiment). Where visible, error bars denote 1 standard error.

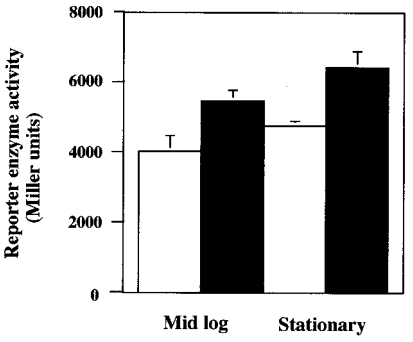

FIG. 3. Expression of *lasI* in response to iron deprivation. Wild-type strain PAO1 was transformed with pPCS223, which contains the *lasI*::*lacZ* reporter fusion (50), and then cultured to mid-log or stationary phase in TSB (open bars) or TSB amended with the iron-specific chelator 2,2-dipyridyl (filled bars). The data represent the average of the means of three independent experiments (two cultures per experiment). Error bars, where visible, represent 1 standard error of the three experimental means. Differences between iron treatments for each culture stage are statistically significantly different ($P = 0.01$).

stationary-phase TSB cultures of the *lasI* mutant JP1, which cannot synthesize PAI-1 (44), and the *lasR* mutant PAO-R1, which lacks the regulatory protein essential to QS (12, 13) (results not shown). All of these observation were consistent with our previous findings, which showed that Mn-SOD production is controlled by QS in this organism (27).

Assessment of iron effects on QS-controlled gene expression was initiated in experiments where either TSB was supplemented with 25 μM $FeCl_3$ or 500 μM 2,2-dipyridyl was added to render the iron unavailable. In wild-type cells cultured to stationary phase in iron-supplemented TSB, no Mn-SOD activity was observed in native gels (results not shown). When 2,2-dipyridyl was included in the culture medium, Mn-SOD was evident in mid-log-phase and stationary-phase PAO1 cells (Fig. 1B), but only in stationary phase in the *lasI* mutant PAO-JP1 (Fig. 1C) and the *lasR* mutant PAO-R1 (results not shown). The iron-sensitive Mn-SOD activity in PAO1 is consistent with prior work demonstrating iron-dependent *sodA* expression (23–25) and demonstrated that either excess or limiting iron can override QS control of *sodA* expression in *P. aeruginosa*.

To establish whether the iron effect on Mn-SOD levels occurs at the transcriptional level, a plasmid containing a *sodA*::*lacZ* transcriptional fusion was transformed into PAO1, PAO-JP1, and PAO-R1, and the above experiments were repeated. As shown in Fig. 2, iron limitation increased *sodA* expression in PAO1 mid-log-phase cells. Iron limitation also caused equivalent levels of *sodA* up-regulation in mid-log-phase cells of PAO-JP1 (Fig. 2), even though the isozyme was not apparent in activity stains of JP1 cell extracts (Fig. 1C). Reporter gene activity was also increased in iron-starved mid-log-phase cells of PAO-R1, but the increase was smaller than in strain PAO1 or PAO-JP1 (Fig. 2). In stationary-phase cells, up-regulation of *sodA* in response to iron starvation also occurred. β-Galactosidase reporter enzyme levels measured in PAO1, PAO-JP1, and PAO-R1 were approximately 4-, 11-, and 7-fold greater, respectively, under iron-limiting growth conditions compared to cells with ample iron. The reporter gene results with both mid-log and stationary-phase cells again demonstrated that QS control of *sodA* could be overridden by the iron starvation response but also showed that the autoinducer PAI-1 and the regulatory protein LasR were still required for maximal *sodA* expression, regardless of iron availability. Further, these studies suggested that Mn-SOD activity in the *lasI* mutant was attenuated by abnormal posttranscriptional activity because iron starvation-based induction of *sodA* in mid-log-phase cells did not result in increased Mn-SOD levels in native gel SOD activity stains.

Effects of iron concentration on *lasI* expression. Additional experiments were conducted to determine if the iron starvation effect on *sodA* expression could be linked to *las* gene expression. Over three independent experiments, iron limitation increased *lasI* expression in the wild-type strain by approximately 30 to 35% (Fig. 3). The increase, while relatively small, was highly reproducible and statistically significant. However, no increase in *lasI*::*lacZ* reporter activity (range, 90 to 150 Miller units) could be measured under the same culture conditions for the *lasI* mutant and the *lasR* mutant (results not shown), implying again that the iron-based response appeared to require LasR and PAI-1 for the maximal effect to be observed. The lack of an iron effect on *lasI* induction in the $LasI^-$ mutant also demonstrated that potential alternative autoinducers were most likely not participating in the iron stress response under the conditions used for cell culture in these experiments.

Production of Mn-SOD in biofilms. An additional important motivation for this study was to investigate potential differences in gene expression and regulation between biofilm cells and planktonic cells. The combination of mutants and the use of Mn-SOD as a native reporter enzyme provided an opportunity to assess the same regulatory issues when the cells were grown as biofilms and to determine if protein expression patterns were different. Surprisingly different Mn-SOD expression patterns were encountered between wild-type and QS mutant biofilms. Mn-SOD was observed only in mature (6-day) PAO1 biofilms (Fig. 4A), whereas it was expressed within the first

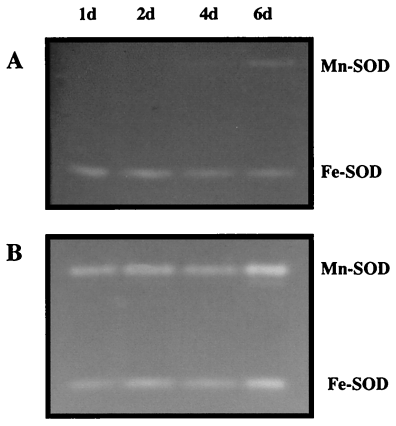

FIG. 4. SOD activity stains of cell extracts (50 μg of protein per lane) of the wild-type strain PAO1 (A) and the *lasI* mutant PAO-JP1 (B) obtained from biofilms cultured with 1/10 TSB for up to 6 days (6d). The data are representative of duplicate experiments, and locations of the Mn-SOD and Fe-SOD bands are as shown.

24 h of *lasI* mutant biofilm formation (Fig. 4B). In identical biofilm experiments with the *lasR* mutant, Mn-SOD levels were the same as observed with the *lasI* mutant (results not shown). Additional biofilm experiments were then conducted to assess whether biofilm cells would respond to iron manipulation as was observed with planktonic cultures. When 2,2-dipyridyl was included in the biofilm flow medium, Mn-SOD was evident in PAO1 biofilms; Mn-SOD activity stains obtained from 24-h biofilm cell extracts appeared weak, but by day 2, they were roughly equivalent to the *lasI* and *lasR* mutant biofilms (Fig. 5) in the other experiments. Given the apparent constitutive *sodA* expression in *lasI* and *lasR* biofilms, further biofilm experiments sought to establish whether *sodA* expression in JP1 and PAO-R1 biofilms was still sensitive to environmental iron concentrations. This was confirmed by supplementing the biofilm medium with iron (25 μM $FeCl_3$). When iron was provided at such ample levels, Mn-SOD was absent in biofilms of wild-type and both mutant strains for the duration of the experiments (up to 6 days [results not shown]). During the course of all biofilm experiments, no apparent changes in the Fe-SOD activity band occurred (results not shown).

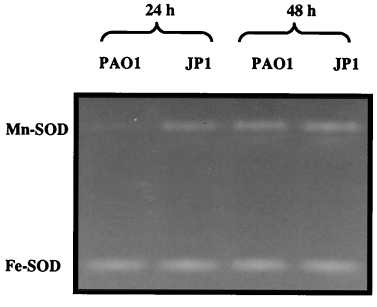

FIG. 5. Mn-SOD expression in PAO1 biofilms as affected by iron starvation. SOD activity was detected using activity stains as described in Materials and Methods. Cell extracts (50 μg per lane) were obtained from PAO1 biofilms grown for 1 or 2 days in 1/10 TSB containing the iron-specific chelator 2,2-dipyridyl and from 1- and 2-day PAO-JP1 biofilms grown in 1/10 TSB. The data are representative of duplicate experiments, and locations of the Mn-SOD and Fe-SOD bands are as shown.

DISCUSSION

Nutritional override of QS. It is clear that HSLs are critical for activation of the QS regulatory circuitry in *P. aeruginosa*. However, prior to the discovery of QS in this organism, the production of several gene products now known to be QS regulated was initially found to be up-regulated by nutritional factors. An example of overlap between a nutrient limitation response and QS-regulated activity in *P. aeruginosa* is pyocyanin production. This exoproduct is typically observed in stationary-phase cells and is controlled by QS (4) but can also be induced by phosphate starvation (20). The effects of other nutrients such as iron have been observed more frequently. Elastase synthesis has been shown to be QS controlled (41, 53); however, its production occurs maximally when *P. aeruginosa* is iron limited but is restricted in the presence of iron (1, 32, 49). Expression of *sodA* was also previously shown to be iron regulated (23–25) and then later found to be controlled by QS (27). Reporter experiments in the present study established that *sodA* can be induced by iron limitation in either log-phase or stationary-phase cells of the wild-type strain (Fig. 1A, 1B, and 2) and QS mutants (Fig. 2). However, relative to the wild-type strain, maximal iron stress-dependent *sodA* induction was much lower in mid-log-phase *lasR* cells and in stationary-phase cells of both *las* mutants (Fig. 2). This implies that the autoinducer PAI-1 and the LasR regulatory protein were still required for maximum iron-starved *sodA* induction under these experimental conditions. This was particularly apparent in stationary-phase cells, where QS is thought to be most active in gene regulation. It is perhaps important that even though relatively quite low, *sodA*::*lacZ* expression in the $LasR^-$ and $LasI^-$ mutants was still inducible by iron starvation (Fig. 2). This suggests the possibility that QS-targeted promoters such as that governing the *fagA-fumC-orfX-sodA* operon can still be binding targets for RNA polymerase in the absence of positive regulatory complexes (such as LasR–PAI-1), if the repressor protein is also not present.

In addition, we bring attention to the observation that apparent wild-type *sodA* transcription occurred in mid-log-phase cells of the $LasI^-$ mutant (Fig. 2), but it did not translate into mature Mn-SOD enzyme as measured by activity stains in native gels (Fig. 1C). While activity stains in native gels are only semiquantitative, cell extract sample loading in these experiments was adequate to detect meaningful SOD enzyme levels, provided for reasonable strain and growth condition comparisons, and allowed for sensitive detection of this isozyme in a wild-type background. This basic observation implies that posttranscriptional processing of the *sodA* mRNA was altered in the *las* mutants. Further, it suggests that LasI, or the autoinducer it synthesizes (PAI-1), is involved in some as yet unknown but essential cellular activity at low cell densities.

The overall iron effect on *lasI* expression (Fig. 3) was small relative to increases in *lasI* transcription previously observed in stationary-phase cells (35), but we note that the increase was highly reproducible and thus directly links the iron stress response with QS circuitry. Integration of QS with nutrient availability should not be unexpected, and it is perhaps no coinci-

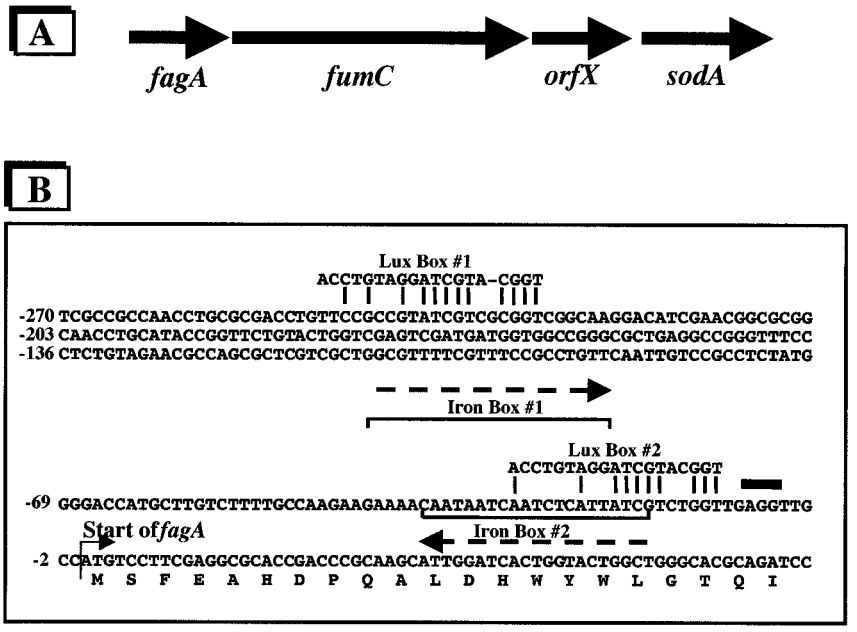

FIG. 6. A potential mechanism for dual control of *sodA* by iron-sensitive and QS circuitry. (A) Gene arrangement of the *fagA-fumC-orfX-sodA* operon. (B) DNA sequence directly upstream of *fagA*, which is promoter proximal in the depicted operon (24, 25). The Fur regulatory protein (encoded by *fur*, which is itself regulated by iron availability) utilizes Fe^{2+} as a corepressor (23). The Fe(II)-Fur complex directly binds to the promoter region of genes that contain a specific regulatory sequence known as the iron box (9). Sequence analysis suggests the presence of iron boxes (orientation of each shown as a dashed line), which is the binding site for Fe(II)-Fur. Note that Mn-SOD production is elevated in *fur* mutants (25). Iron boxes are located at nucleotide positions -18 to -37 and -21 to -42. Positions of putative Lux boxes, the binding site for the PAI-1–LasR complex, are also shown at -11 to -29 and at -228 to -247. Nucleotide positions that are homologous with the consensus *Vibrio* Lux box are indicated by the connecting lines. Under high iron conditions, binding of the iron box by the Fe(II)-Fur complex would inhibit the LasR–PAI-1 complex from binding to the Lux box 2 region and would also inhibit transcription possibly originating upstream due to potential activation from binding at Lux box 1. Upon iron starvation, the Fe(II)-Fur complex would not be present, and thus the LasR–PAI-1 complex would be free to activate transcription. The bold thick line indicates a potential ribosome binding site.

dence that QS-based regulation is most often observed in stationary-phase cultures when cell densities approach levels that represent a significant nutrient sink and indeed growth rates are declining due to limitation of some nutrient(s). Nutrients such as iron are subject to biotic and abiotic redox reactions, typically yielding insoluble precipitates under aerobic conditions. Thus, the bioavailability of such nutrients can be sparingly low regardless of local biological demand. In biofilms where cell densities can approach 10^{10} to 10^{12} cells per cm^3 (6), low nutrient bioavailability coupled with high localized nutrient demand could result in a nutrient stress response. Lazazzera (36) has recently reviewed a similar concept for *Bacillus subtilis*, and there are other reports that suggest QS and nutrient sensing in gram-negative bacteria are integrated. Kjelleberg and colleagues have connected carbon starvation, QS, and the stringent response in a marine *Vibrio* isolate (14, 16, 39, 50). Other, similar interactions integrating nutrient limitation, cell-to-cell communication, and the stringent response have also been reported for *Myxococcus xanthus* (19, 33, 34, 48) and thus serve to further demonstrate the complexity of cell-to-cell signaling systems. When possible strategies for manipulating QS for controlling bacterial infections are considered, the effects of multiple control mechanisms or nutritionally based regulatory override systems must be recognized. The onset of QS-regulated gene expression relative to the accumulation of autoinducers or to other metabolites, and the timing of their accumulation relative to changes in the cell nutritional state, represents a critical area of research. To account for the observations made in this work, we examined the promoter region of the operon that contains *sodA* (Fig. 6). Iron boxes (Fur-Fe^{2+} binding sites [24]) and putative LasR–PAI-1 binding sites (designated Lux boxes) were found and are located such that under high iron conditions, the Fur repressor protein would bind as a dimer to the iron boxes, inhibiting binding and thereby reducing transcriptional activation by the LasR–PAI-1 complex. The occurrence of two (or more) regulatory elements in the promoter region of other QS-regulated genes has not been studied but represents an important issue for understanding and manipulating QS in bacteria.

Biofilm gene expression. Another important issue addressed in this study included *P. aeruginosa* biofilm cell physiology. The use of defined regulatory mutants and an endogenous reporter enzyme that had been shown to be controlled by both QS and nutritional effects presented an opportunity to assess basic protein expression in biofilms and make relevant comparisons to planktonic cells. *P. aeruginosa* has been used as a model organism for studying biofilm behavior and for the development of biofilm control strategies (7), and though recent progress has been significant, *P. aeruginosa* biofilm cell physiology is still only poorly understood. Particularly lacking is information regarding gene expression and regulation. Gradients in metabolic activity have been shown to exist in *P. aerugi-*

nosa biofilms, and some information regarding adaptive gene regulation has also been recently published (31). These studies examined *P. aeruginosa* gene regulation in response to environmental stress, showing, for example, that induction of *phoA* in response to phosphate limitation occurred only in the upper region (ca. 20%) of the biofilm (31).

In the present study, differences in Mn-SOD levels between wild-type and QS mutant biofilms were significant. Whereas Mn-SOD activity was not detected in wild-type biofilms until after 6 days, it was detectable within the first 24 h of biofilm formation by both *las* mutants. The notable lack of Mn-SOD in wild-type biofilms implies that the cells were not limited for iron and is consistent with an analysis of iron availability under these growth conditions. Chemical analysis of TSB showed an iron concentration of 15 μM. The iron content in the 1/10 TSB used for biofilm experiments would be proportionately lower, but the constant flow would continuously deliver fresh medium over the developing biofilm. Over the course of such experiments, the total iron made available to the developing biofilm would exceed batch conditions at least eightfold, while total biomass generated in the different growth modes would be similar (T. R. McDermott and D. J. Hassett, unpublished data). The appearance of Mn-SOD in 6-day-old wild-type biofilms likely represents iron limitation resulting from the reduced iron in 1/10 TSB being insufficient for the high cell numbers accumulated during this time period ($\sim 5.0 \times 10^{10}$ total on the stainless steel slide) and/or potential diffusion limitations.

The constitutive presence of Mn-SOD in the *las* mutant biofilms reveals a potentially important behavior difference between wild-type and *las* mutant cells and an as yet undefined (but not necessarily unexpected) important characteristic of QS regulation. As implied by the Mn-SOD activity in the *las* mutant biofilms, the absence of normal QS regulatory components resulted in the apparent unrestricted expression of *sodA*, suggesting that in biofilm cells QS regulation may exert negative gene control. An alternative explanation is that developmentally immature biofilms have an increased iron requirement that results in an iron stress response. However, adding the iron-specific chelator 2,2-dipyridyl to the medium of wild-type biofilms resulted in the induction of Mn-SOD activity, implying that such biofilms were not otherwise iron limited (also see above). However, there are other potentially important issues to consider in assessing the constitutive expression of Mn-SOD in *lasI* mutant biofilms. There are alternative transcriptional start sites within *fagA* and *fumC* (24) that could result in *sodA* expression and represent biofilm-specific gene regulation, and it has been established that synthesis of the *P. aeruginosa* iron chelator pyoverdine is responsive to iron availability (46) and also under QS control (51). In the biofilm experiments conducted with the *lasI* mutant, lack of the iron chelator pyoverdine could conceivably result in significantly reduced iron acquisition by the mutant and thus result in an iron starvation-like response. Regardless, the constitutive presence of Mn-SOD in *las* mutant biofilms suggests that a biofilm-specific regulatory system that is independent of the Las system is present and can affect gene expression in the absence of LasR or PAI-1. This may be an important consideration when evaluating autoinducer analogs as an alternative chemotherapy for biofilm-related infections.

Of additional importance, the experiments in this study demonstrated that biofilm cells respond like planktonic cells to environmental effects—in this case, iron concentration. The addition of iron to the biofilm medium caused *las* mutant biofilms to predictably repress Mn-SOD levels, and likewise the addition of 2,2-dipyridyl to the medium caused wild-type biofilm cells to up-regulate Mn-SOD activity (Fig. 5). This suggests that at least in the case of *sodA* control, basic iron sensor-response circuitry is unchanged for cells growing in either setting. The basis for the apparent up-regulation of *sodA* in the QS mutant biofilms under conditions that are not obviously iron limiting is the focus of continuing experiments.

In summary, this study demonstrated that (i) the nutritional status of the cell must be taken into account when evaluating QS-based gene expression, (ii) QS-based gene regulation models based on studies with planktonic cells must be modified in order to explain biofilm gene expression behavior, and (iii) gene expression in biofilms is dynamic. In addition, the results from the *las* mutant biofilms implied that QS regulation can exert negative regulatory control. Determining physiological differences between biofilms and planktonic cultures is critical to the understanding and eventual treatment of *P. aeruginosa* infections such as that found in the cystic fibrosis lung or for removing problematic biofilms from industrial or environmental settings. Previous studies showed evidence that bacteria in lung tissue grow under iron-limited conditions (5, 10, 18). Further experiments aimed at understanding in situ conditions and gene expression, particularly as related to *P. aeruginosa* infections, should offer significant opportunity to improve our understanding of disease and its control.

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REFERENCES

1. **Bjorn, M. J., P. A. Sokol, and B. H. Iglewski.** 1979. Influence of iron on yields of extracellular products in *Pseudomonas aeruginosa* cultures. J. Bacteriol. **138:**193–200.
2. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
3. **Braun, V., S. Schaffer, K. Hantke, and W. Troger.** 1990. Regulation of gene expression by iron, p. 164–179. *In* G. Hauka and R. Thauer (ed.), The molecular basis of bacterial metabolism. Springer-Verlag, Heidelberg, Germany.
4. **Brint, J. M., and D. E. Ohman.** 1995. Synthesis of multiple exoproducts in *Pseudomonas aeruginosa* is under the control of RhlR-RhlI, another set of regulators in strain PAO1 with homology to the autoinducer-responsive LuxR-LuxI family. J. Bacteriol. **177:**7155–7163.
5. **Brown, M. R. W., H. Anwar, and P. A. Lambert.** 1984. Evidence that mucoid *Pseudomonas aeruginosa* in the cystic fibrosis lung grows under iron-restricted conditions. FEMS Microbiol. Lett. **21:**113–117.
6. **Characklis, W. G., K. C. Marshall, and G. A. McFeters.** 1990. The microbial cell, p. 131–160. *In* W. G. Characklis and K. C. Marshall (ed.), Biofilms. John Wiley & Sons, New York, N.Y.
7. **Costerton, J. W., Z. Lewandowski, D. E. Caldwell, D. R. Korbe, and H. M. Lappin-Scott.** 1995. Microbial biofilms. Annu. Rev. Microbiol. **49:**711–745.
8. **Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg.** 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science **280:**295–298.
9. **de Lorenzo, V., S. Wee, M. Herrero, and J. B. Neilands.** 1987. Operator sequences of the aerobactin operon of plasmid ColV-K30 binding the ferric uptake regulation (*fur*) repressor. J. Bacteriol. **169:**2624–2630.
10. **Emery, T.** 1982. Iron metabolism in humans and plants. Am. Sci. **70:**626–631.
11. **Fuqua, C., S. C. Winans, and E. P. Greenberg.** 1996. Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcrip-

tional regulators. Annu. Rev. Microbiol. **50:**727–51.
12. **Gambello, M. J., and B. H. Iglewski.** 1991. Cloning and characterization of the *Pseudomonas aeruginosa lasR* gene: a transcriptional activator of elastase expression. J. Bacteriol. **173:**3000–3009.
13. **Gambello, M. J., S. Kaye, and B. H. Iglewski.** 1993. LasR of *Pseudomoas aeruginosa* is a transcriptional activator of the alkaline protease gene (*apr*) and an enhancer of exotoxin A expression. Infect. Immun. **61:**1180–1184.
14. **Givskov, M., R. de Nys, M. Manefield, L. Gram, R. Maximilien, L. Eberl, S. Molin, P. D. Steinberg, and S. Kjelleberg.** 1996. Eukaryotic interference with homoserine lactone-mediated prokaryotic signaling. J. Bacteriol. **178:**6618–6622.
15. **Govan, J. R. W., and V. Deretic.** 1996. Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Microbiol. Rev. **60:**539–574.
16. **Gram, L., R. de Nys, R. Maximillen, M. Givskov, P. D. Steinberg, and S. Kjelleberg.** 1996. Inhibitory effects of secondary metabolites from the red alga *Delisea pulchra* on swarming mobility of *Proteus mirabilis*. Appl. Environ. Microbiol. **62:**4284–4287.
17. **Greenberg, E. P.** 1997. Quorum sensing in gram-negative bacteria. ASM News **63:**371–377.
18. **Haas, B., J. Kraut, J. Marks, S. C. Zanker, and D. Castigenetti.** 1991. Siderophore presence in sputa of cystic fibrosis patients. Infect. Immun. **59:**3997–4000.
19. **Harris, B. Z., D. Kaiser, and M. Singer.** 1998. The guanosine nucleotide (p)ppGpp initiates development and A-factor production in *Myxococcus xanthus*. Genet. Dev. **12:**1022–1035.
20. **Hassett, D. J., L. Charniga, K. A. Bean, D. E. Ohman, and M. S. Cohen.** 1992. Response of *Pseudomonas aeruginosa* to pyocyanin: mechanisms of resistance, antioxidant defenses, and demonstration of a manganese-cofactored superoxide dismutase. Infect. Immun. **60:**328–336.
21. **Hassett, D. J., W. A. Woodruff, D. J. Wozniak, M. L. Vasil, M. S. Cohen, and D. E. Ohman.** 1993. Cloning and characterization of the *Pseudomonas aeruginosa sodA* and *sodB* genes encoding manganese- and iron-cofactored superoxide dismutase: demonstration of increased manganese superoxide dismutase activity in alginate-producing bacteria. J. Bacteriol. **175:**7658–7665.
22. **Hassett, D. J., H. P. Schweizer, and D. E. Ohman.** 1995. *Pseudomonas aeruginosa sodA* and *sodB* mutants defective in manganese- and iron-cofactored superoxide dismutase activity demonstrate the importance of the iron-cofactored form in aerobic metabolism. J. Bacteriol. **177:**6330–6337.
23. **Hassett, D. J., P. Sokol, M. L. Howell, J.-F. Ma, H. P. Schweizer, U. Ochsner, and M. L. Vasil.** 1996. Ferric uptake regulator (Fur) mutants of *Pseudomonas aeruginosa* demonstrate defective siderophore-mediated iron uptake and altered aerobic metabolism. J. Bacteriol. **178:**3996–4003.
24. **Hassett, D. J., M. L. Howell, U. Ochsner, M. Vasil, Z. Johnson, and G. E. Dean.** 1997. An operon containing *fumC* and *sodA* encoding fumarase C and manganese superoxide dismutase is controlled by the ferric uptake regulator (Fur) in *Pseudomonas aeruginosa*: *fur* mutants produce elevated alginate levels. J. Bacteriol. **179:**1452–1459.
25. **Hassett, D. J., M. L. Howell, P. A. Sokol, M. L. Vasil, and G. E. Dean.** 1997. Fumarase C activity is elevated in response to iron deprivation and in mucoid, alginate-producing *Pseudomonas aeruginosa*: cloning and characterization of *fumC* and purification of the native FumC. J. Bacteriol. **179:**1442–1450.
26. **Hassett, D. J., J. G. Elkins, D.-J. Ma, and T. R. McDermott.** 1999. *Pseudomonas aeruginosa* biofilm sensitivity to biocides: use of hydrogen peroxide as a model antimicrobial agent for examining resistance mechanisms. Methods Enzymol. **310:**599–608.
27. **Hassett, D. J., J.-F. Ma, J. G. Elkins, T. R. McDermott, U. A. Ochsner, S. E. H. West, C.-T. Huang, J. Fredericks, S. Burnett, P. S. Stewart, G. McFeters, L. Passador, and B. H. Iglewski.** 1999. Quorum sensing in *Pseudomonas aeruginosa* controls expression of catalase and superoxide dismutase genes and mediates biofilm susceptibility to hydrogen peroxide. Mol. Microbiol. **34:**1082–1093.
28. **Hassett, D. J., U. A. Ochsner, T. de Kievit, B. H. Iglewski, L. Passador, T. S. Livinghouse, J. A. Whitsett, and T. R. McDermott.** Genetics of quorum sensing circuitry in *Pseudomonas aeruginosa*: implications for control of pathogenesis, biofilm formation and antibiotic/biocide resistance. *In* U. Streips and R. Yasbin (ed.), Modern molecular genetics, in press. Wiley-Liss, New York, N.Y.
29. **Holden, M. T., S. Ram Chhabra, R. de Nys, P. Stead, N. J. Bainton, P. J. Hill, M. Manefield, N. Kumar, M. Labatte, D. England, S. Rice, M. Givskov, G. P. Salmond, G. S. Stewart, B. W. Bycroft, S. Kjelleberg, and P. Williams.** 1999. Quorum-sensing cross talk: isolation and chemical characterization of cyclic dipeptides from *Pseudomonas aeruginosa* and other gram-negative bacteria. Mol. Microbiol. **33:**1254–1266.
30. **Holloway, B. W.** 1969. Genetics of *Pseudomonas*. Bacteriol. Rev. **33:**419–443.
31. **Huang, C.-T., K. D. Xu, G. A. McFeters, and P. A. Stewart.** 1998. Spatial patterns of alkaline phosphatase expression within bacterial colonies and biofilm in response to phosphate starvation. Appl. Environ. Microbiol. **64:**1526–1531.
32. **Iglewski, B. H., L. Rust, and R. Bever.** 1990. Molecular analysis of *Pseudomonads* elastase, p. 36–53. *In* S. Silver, A. M. Chakrabarty, B. H. Iglewski, and S. Kaplan (ed.), *Pseudomonas:* biotransformations, pathogenesis, and evolving biotechnology. American Society for Microbiology, Washington, D.C.
33. **Kuspa, A., L. Kroos, and D. Kaiser.** 1986. Intercellular signaling is required for developmental gene expression in *Myxococcus xanthus*. Dev. Biol. **117:**267–276.
34. **Kuspa, A., L. Plamann, and D. Kaiser.** 1992. Identification of heat-stable A-factor from *Myxococcus xanthus*. J. Bacteriol. **174:**3319–3326.
35. **Latifi, A., M. Foglino, K. Tanaka, P. Williams, and A. Lazdunski.** 1996. A hierarchial quorum-sensing cascade in *Pseudomonas aeruginosa* links the transcriptional activators LasR and RhlR (VsmR) to expression of the stationary-phase sigma factor RpoS. Mol. Microbiol. **21:**1137–1146.
36. **Lazazzera, B. A.** 2000. Quorum sensing and starvation: signals for entry into stationary phase. Curr. Opin. Microbiol. **3:**177–182.
37. **Mekalanos, J. J.** 1992. Environmental signals controlling expression of virulence determinants in bacteria. J. Bacteriol. **174:**1–7.
38. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
39. **Östling, J., K. Flärdh, and S. Kjelleberg.** 1995. Isolation of a carbon starvation regulatory mutant in a marine vibrio. J. Bacteriol. **177:**6978–6982.
40. **Östling, J., L. Holmquist, and S. Kjelleberg.** 1996. Global analysis of the carbon starvation response of a marine vibrio disrupted in genes homologous to *relA* and *spoT*. J. Bacteriol. **178:**4901–4908.
41. **Passador, L., J. M. Cook, M. J. Gambello, L. Rust, and B. H. Iglewski.** 1993. Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. Science **260:**1127–1130.
42. **Pearson, J. P., K. M. Gray, L. Passador, K. D. Tucker, A. Eberhard, B. H. Iglewski, and E. P. Greenberg.** 1994. Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. Proc. Natl. Acad. Sci. USA **91:**197–201.
43. **Pearson, J. P., L. Passador, B. H. Iglewski, and E. P. Greenberg.** 1995. A second N-acylhomoserine lactone produced by *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **92:**1490–1494.
44. **Pearson, J. P., C. P. Everett, and B. H. Iglewski.** 1997. Roles of *Pseudomonas aeruginosa las* and *rhl* quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. J. Bacteriol. **179:**5756–5767.
45. **Pesci, E. C., J. B. Milbank, J. P. Pearson, S. McKnight, A. S. Kende, E. P. Greenberg, and B. H. Iglewski.** 1999. Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **96:**11229–11234.
46. **Rombel, I. T., B. J. McMorran, and I. L. Lamont.** 1995. Identification of a DNA sequence motif required for expression of iron-regulated genes in pseudomonads. Mol. Gen. Genet. **246:**519–528.
47. **Sambrook, J., E. F. Fristch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
48. **Singer, M., and D. Kaiser.** 1995. Ectopic production of guanosine penta- and tetraphosphate can initiate early developmental gene expression in *Myxococcus xanthus*. Genes Dev. **9:**1633–1644.
49. **Sokol, P. A., C. D. Cox, and B. H. Iglewski.** 1982. *Pseudomonas aeruginosa* mutants altered in their sensitivity to the effect of iron on toxin A or elastase yields. J. Bacteriol. **151:**783–787.
50. **Srinivasan, S., J. Ostling, T. Charlton, R. de Nys, K. Takayama, and S. Kjellberg.** 1998. Extracellular signal molecule(s) involved in the carbon starvation response of marine *Vibrio* sp. strain S14. J. Bacteriol. **180:**201–209.
51. **Van Delden, C., E. C. Pesci, J. P. Pearson, and B. H. Iglewski.** 1998. Starvation selection restores elastase and rhamnolipid production in a *Pseudomonas aeruginosa* quorum-sensing mutant. Infect. Immun. **66:**4499–4502.
52. **Whiteley, M., K. M. Lee, and E. P. Greenberg.** 1999. Identification of genes controlled by quorum sensing in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **96:**13904–13909.
53. **Winson, M. K., M. Camara, A. Latifi, M. Foglino, S. R. Chhabra, M. Daykin, M. Bally, V. Chapon, G. P. Salmond, B. W. Bycroft, A. Lazdunski, G. S. A. B. Stewart, and P. Williams.** 1995. Multiple N-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **92:**9427–9431.