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# Quorum sensing in *Pseudomonas aeruginosa* controls expression of catalase and superoxide dismutase genes and mediates biofilm susceptibility to hydrogen peroxide

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## Summary

**Quorum sensing (QS) governs the production of virulence factors and the architecture and sodium dodecyl sulphate (SDS) resistance of biofilm-grown *Pseudomonas aeruginosa*. *P. aeruginosa* QS requires two transcriptional activator proteins known as LasR and RhlR and their cognate autoinducers PAI-1 (*N*-(3-oxododecanoyl)-L-homoserine lactone) and PAI-2 (*N*-butyryl-L-homoserine lactone) respectively. This study provides evidence of QS control of genes essential for relieving oxidative stress. Mutants devoid of one or both autoinducers were more sensitive to hydrogen peroxide and phenazine methosulphate, and some PAI mutant strains also demonstrated decreased expression of two superoxide dismutases (SODs), Mn-SOD and Fe-SOD, and the major catalase, KatA. The expression of *sodA* (encoding Mn-SOD) was particularly dependent on PAI-1, whereas the influence of autoinducers on Fe-SOD and KatA levels was also apparent but not to the degree observed with Mn-SOD. β-Galactosidase reporter fusion results were in agreement with these findings. Also, the addition of both PAIs to suspensions of the PAI-1/2-deficient double mutant partially restored KatA activity, while the addition of PAI-1 only was sufficient for full restoration of Mn-SOD activity. In biofilm studies, catalase activity in wild-type bacteria was significantly reduced relative to planktonic bacteria; catalase activity in the PAI mutants was reduced even further and consistent with relative differences observed between each strain grown planktonically. While wild-type and mutant biofilms contained less catalase activity, they were more resistant to hydrogen peroxide treatment than their respective planktonic counterparts. Also, while catalase was implicated as an important factor in biofilm resistance to hydrogen peroxide insult, other unknown factors seemed potentially important, as PAI mutant biofilm sensitivity appeared not to be incrementally correlated to catalase levels.**

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## Introduction

*Pseudomonas aeruginosa* is a Gram-negative bacterium that occupies many diverse niches. It is an opportunistic pathogen of humans (e.g. cystic fibrosis or immunocompromised) and plants (Govan and Harris, 1986), but is also a significant problem in environmental and industrial settings because it can be a primary component of bacterial biofilms (Costerton *et al*., 1994). Within such diverse niches, *P. aeruginosa* adapts to each microenvironment, in part through a process called quorum sensing (QS; for review, see Fuqua *et al*., 1996). As so far elucidated, QS in *P. aeruginosa* is governed by two gene tandems, *lasR-lasI* and *rhlRrhlI* (Passador *et al*., 1993; Pearson *et al*., 1994; 1995). Transcription of these genes is maximal in early stationary phase, where cell density is high. The

*las* system is composed of LasR, a positive transcriptional activator protein, and LasI, which catalytically produces one of two known *Pseudomonas* autoinducers [PAI-1; *N*-(3-oxododecanoyl)-L-homoserine lactone]. The second tier consists of RhlR, which, like LasR, is a transcriptional activator, and RhlI, which produces the second autoinducer, PAI-2 [*N*-butyl-L-homoserine lactone] (Jiang *et al*., 1998). To date, the *las* and *rhl* QS systems have been shown to activate the expression of a myriad of genes, many of which are involved in virulence (Brint and Ohman, 1995; Winson *et al*., 1995; Latifi *et al*., 1996; Pesci *et al*., 1997; Reimmann *et al*., 1997). More recently, QS has been implicated in the differentiation, architecture and sodium dodecyl sulphate (SDS) resistance of *P. aeruginosa* biofilms (Davies *et al*., 1998).

Metabolic processes in *P. aeruginosa* are most efficient during aerobic respiration. However, two hazardous by-products of aerobic respiration are the superoxide anion ($O_2^-$) and hydrogen peroxide ($H_2O_2$). To reduce the potential hazards of these reactive intermediates, the organism possesses two superoxide dismutases (SODs; Hassett *et al*., 1992; 1993; 1995) cofactored by iron (Fe) or manganese (Mn) and two haem-containing catalases (KatA and KatB; Hassett *et al*., 1992; Brown *et al*., 1995; Ma *et al*., 1999). Mn-SOD (encoded by *sodA*) is expressed maximally when organisms are deprived of iron (Hassett *et al*., 1995; 1997a,b), while Fe-SOD (encoded by *sodB*) activity is maximal when iron is plentiful (Hassett *et al*., 1992). KatA and, in particular, KatB activities are increased when bacteria are exposed to $H_2O_2$ (Brown *et al*., 1995). While the above environmental factors are known to influence the expression of *sodA*, *sodB*, *katA* or *katB*, the regulatory circuitry governing the expression of these genes is unknown.

In this study, we demonstrate that PAIs are necessary for optimal resistance to $H_2O_2$ and the $O_2^-$-generating agent phenazine methosulphate. PAI-1 was found to be essential for optimal transcription of the operon containing the *sodA* gene, while optimal *sodB* transcription required PAI-2. Control of *katA* appeared to be partially mediated by both PAI-1 and PAI-2. Finally, we demonstrate that QS is important in the resistance of *P. aeruginosa* biofilms to the oxidizing biocide $H_2O_2$.

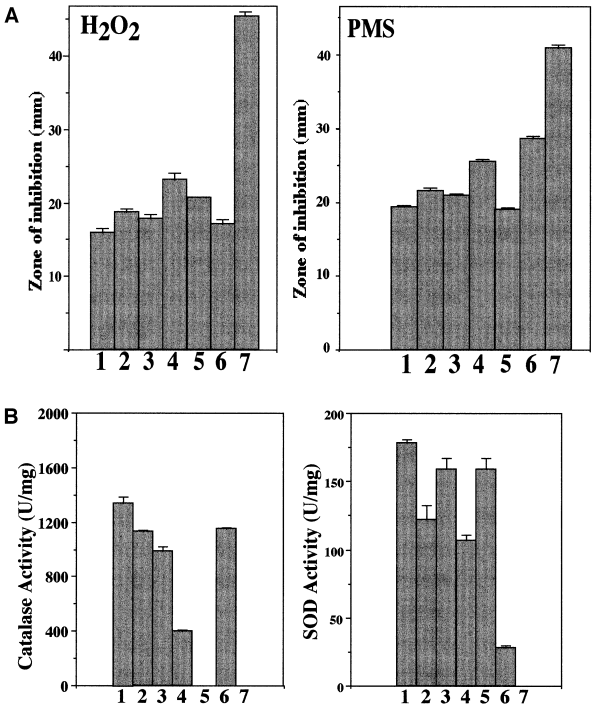


**Fig. 1.** Sensitivity of wild-type and autoinducer mutants to $H_2O_2$ and PMS and catalase and SOD activity.
A. Sensitivity to each agent was recorded as the mean ± SE ($n = 9$) of the diameter of growth inhibition. All values are statistically significant when compared with wild-type values at $P < 0.005$.
B. Cell-free extracts were assayed for SOD and catalase activity, as described in *Experimental procedures*. Results were expressed as total SOD (Fe-SOD + Mn-SOD) and catalase (KatA and KatB) activity. Error bars represent the standard error of the mean ($n = 3$). All values are statistically significant when compared with wild-type values at $P < 0.005$, except for the SOD activity of the *rhlI* that was significant at a level of $P < 0.05$. Lane 1, wild-type strain PAO1; lane 2, PAO-JP1 (*lasI*); lane 3, PDO100 (*rhlI*); lane 4, PAO-JP2 (*lasIrhlI*); lane 5, either a *katA* mutant for $H_2O_2$ sensitivity and catalase assays or a *sodA* mutant for PMS sensitivity and SOD assays; lane 6, either a *katB* mutant for $H_2O_2$ sensitivity and catalase assays or a *sodB* mutant for PMS sensitivity and SOD assays; lane 7, either a *katAkatB* mutant for $H_2O_2$ sensitivity and catalase assays or a *sodAsodB* mutant for PMS sensitivity and SOD assays.

## Results

### *PAI mutants are more sensitive to $H_2O_2$ and phenazine methosulphate and possess decreased catalase and SOD activity*

Genes known to be controlled by the *las* or *rhl* QS systems include *lasI*, *lasB*, *lasA*, *apr*, *toxA*, *rhlI*, *rhlAB* and *rpoS* (Latifi *et al*., 1996; Pesci *et al*., 1997). We postulated that, if QS circuitry participates in the regulation of genes encoding the antioxidants catalase and SOD, then organisms deficient in the production of one or both autoinducers would be more sensitive to reactive oxygen intermediates. To test this hypothesis, we monitored sensitivity of wild-type, *lasI*, *rhlI* and *lasIrhlI* strains to $H_2O_2$ and phenazine methosulphate (PMS), a $O_2^-$-generating agent that can also increase intracellular $H_2O_2$ levels. As shown in Fig. 1A, *lasI* and *rhlI* mutants demonstrated increased sensitivity to both oxidants, while the *lasIrhlI* double mutant was the most sensitive. We also included isogenic mutants lacking one or both catalases or SODs to compare QS-mediated control with a null catalase or SOD phenotype. As expected, a *katB* mutant was slightly

sensitive to $H_2O_2$, while *katA* and *katAkatB* mutants demonstrated the greatest sensitivity. Similarly, the *sodB* and *sodAsodB* mutants were very sensitive to PMS relative to wild-type bacteria, while the *sodA* mutant was not. These results are consistent with our previous observations (Brown *et al*., 1995; Hassett *et al*., 1995; Ma *et al*., 1999). A *katAkatB* mutant was only moderately sensitive to PMS, indicating that its primary product of redox cycling is $O_{2^-}$.

To test whether the enhanced sensitivity of the autoinducer mutants to $H_2O_2$ and PMS might be caused by reduced activities of protective catalase and SOD antioxidant enzymes, these enzymes were assayed in stationary phase cultures, in which both PAI-1 and PAI-2 levels are maximal in the wild-type strain (Pesci *et al*., 1997). As shown in Fig. 1B, catalase activity was highest in the wild-type strain, whereas an absence of PAI-1 (*lasI* mutant), PAI-2 (*rhlI* mutant) or both PAI-1 and PAI-2 was associated with reduced catalase activity. Catalase activity was only moderately reduced in the *katB* mutant but was absent in *katA* and *katAkatB* strains (Ma *et al*., 1999). Autoinducers also appear to play an important role in SOD expression. Total SOD activity was significantly lower ($P < 0.005$) in the mutants carrying the mutated *lasI* allele, but only small reductions were observed in the *rhlI* mutant, which is only affected in PAI-2 synthesis (Fig. 1B). In contrast, inactivation of *sodA* caused only a small reduction in total SOD activity, while nearly 85% was absent in a *sodB* mutant. Not surprisingly, a *sodAsodB* double mutant possessed no detectable SOD activity, consistent with previous observations (Hassett *et al*., 1995).

### *Altered catalase and SOD isozyme profiles of* P. aeruginosa *autoinducer mutants*

As the autoinducer mutants demonstrated greater sensitivity to $H_2O_2$ and PMS, and possessed less catalase and SOD activity, we next determined whether one or both autoinducers played a role(s) in the activation/repression of the different catalase and SOD isozymes. In *P. aeruginosa* cells not challenged with $H_2O_2$ or paraquat, KatB activity is normally either not detected or is expressed only at very low levels, whereas KatA is expressed constitutively, with maximal expression occurring during the transition to stationary phase (Brown *et al*., 1995). Consistent with this normal expression pattern, KatB activity was absent (Fig. 2A). However, the intensity of the KatA activity band in the QS mutants was clearly reduced relative to the wild-type strain and paralleled the decreased activity present in cell extracts of the autoinducer mutants described in Fig. 1B. Likewise, the SOD isozyme profile of the autoinducer mutants revealed marked differences (Fig. 2B). As in previous reports (Hassett *et al*., 1992; 1993; 1995), stationary phase wild-type cells produced both Fe-SOD and, to a lesser extent, Mn-SOD. When examining the different strains in these experiments, the *rhlI* mutant produced slightly less Mn-SOD and about half the Fe-SOD, while Mn-SOD activity appeared to be completely absent in the *lasI* and *lasIrhlI* strains (Fig. 2B).

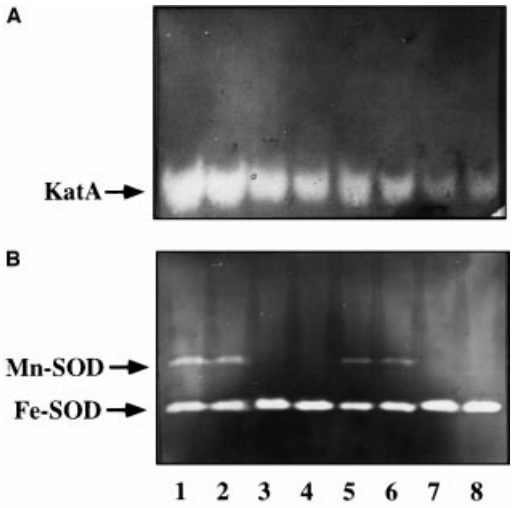


**Fig. 2.** Electrophoretic profile of catalase (A) and SOD (B) isozymes of wild-type and autoinducer mutants. Duplicate cell-free extracts (20 mg of total protein for catalase, 40 mg for SOD) from stationary phase, L broth-grown bacteria were separated by non-denaturing polyacrylamide gel electrophoresis and stained for catalase (Wayne and Diaz, 1986) and SOD (Clare *et al*., 1984) activity. PAO1, lanes 1 and 2; PAO-JP1 (*lasI*), lanes 3 and 4; PDO100 (*rhlI*), lanes 5 and 6; PAO-JP2 (*lasIrhlI*), lanes 7 and 8. Note the reduced Fe- and Mn-SOD activity staining for the *rhlI* mutant, PDO100, results that were supported by linear scanning densitometry.

### *PAI add-back experiments*

To demonstrate that PAI-1 and PAI-2 are required for optimal KatA and SOD activities, wild-type and *lasIrhlI* strains were grown aerobically to stationary phase in the presence of 1 mM PAI-1, 1 mM PAI-2 or 1 mM of both autoinducers. As shown in Fig. 3A, catalase activity in wild-type cells was unaffected by the addition of autoinducers (lanes 1–4). When added individually to the *lasIrhlI* mutant, the effect of either autoinducer on catalase activity was somewhat variable, but tended to enhance catalase activity. However, when added together, they significantly and reproducibly increased total cellular catalase specific activity (lanes 5–8). The addition of 1 mM PAI-1 also restored Mn-SOD activity (Fig. 3B, lanes 4–6; compare with control bacteria, lanes 1–3), while PAI-2 had no effect (lanes 7–9). The addition of both autoinducers allowed for Mn-SOD activity comparable with that seen with PAI-1 alone (lanes 10–12).

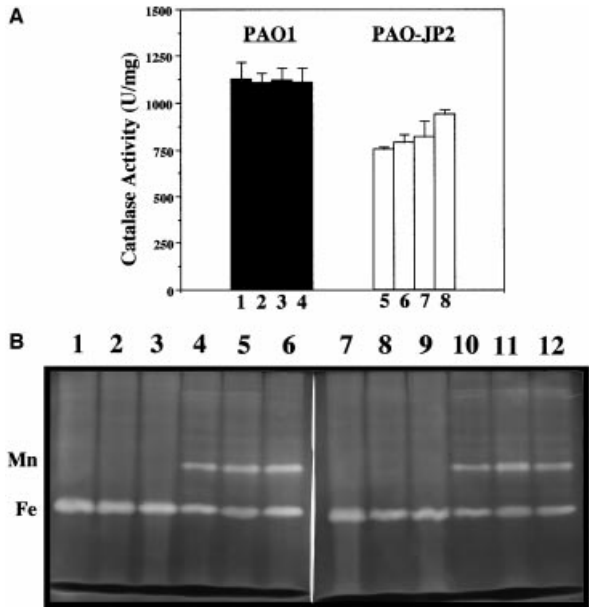


**Fig. 3.** Effect of *P. aeruginosa* autoinducers on catalase and Mn-SOD activity.
A. Wild-type strain PAO1 (lanes 1–4) and *lasIrhlI* strain PAO-JP2 (lanes 5–8) were grown aerobically to stationary phase in L broth containing 0.2% (v/v) ethyl acetate (lanes 1 and 5), 1 mM PAI-1 (lanes 2 and 6), 1 mM PAI-2 (lanes 3 and 7) or 1 mM PAI-1 and PAI-2 (lanes 4 and 8). Cell-free extracts were assayed for catalase activity (Beers and Sizer, 1952). The results are the mean ± SE of three samples.
B. Cell-free extracts from the *lasIrhlI* mutant PAO-JP2 (triplicate samples of 40 mg of total protein) were separated by non-denaturing polyacrylamide gel electrophoresis and stained for SOD activity (Clare *et al*., 1984). Fe and Mn indicates the Fe-SOD and Mn-SOD activity bands respectively. Lanes 1–3, control; lanes 4–6, plus 1 mM PAI-1; lanes 7–9, plus 1 mM PAI-2; lanes 10–12, plus 1 mM PAI-1 and PAI-2.

### *QS control of* sodA*,* sodB *and* katA

To assess the effect of PAIs on the transcription of *sodA*, *sodB*, *katA* and *katB*, we constructed *lacZ* reporter fusions for each gene. Expression of *sodA* was studied with a *sodA::lacZ* fusion plasmid that contained the entire Fur-regulated operon, including the upstream genes *fagA* (untranslated RNA of unknown function, unpublished), *fumC* (fumarase C) and *orfX* (unknown function; Hassett *et al*., 1997a,b), with the primary transcript initiating upstream of *fagA* (Hassett *et al*., 1997a). As shown in Fig. 4A, *sodA* expression was significantly reduced in the *lasI* mutant and is consistent with the requirement of PAI-1 for expression (Fig. 3B). A *lasR* mutant also showed a dramatic reduction in *sodA* expression (data not shown). Reduced *sodA* expression was also evident in the *rhlI* mutant and the *lasIrhlI* double mutant. Apparent transcription of *sodB* was essentially at wild-type levels in the *lasI* and *lasIrhlI* mutants, but was reduced ≈ 50% in the *rhlI* mutant (Fig. 4B). This is consistent with the SOD activity gel data in Fig. 2B. Finally, *katA::lacZ* activity was lower in both *lasI* and *rhlI* strains, with the maximal reduction observed in the *lasIrhlI* mutant (Fig. 4C). Reporter activity using a *katB::lacZ* fusion plasmid in $H_2O_2$- treated and control bacteria was not significantly affected by QS circuitry (data not shown).

### *Planktonic cells versus biofilm* $H_2O_2$ *susceptibility*

In related experiments, we noted that the effects of the *lasI* and *rhlI* mutations on catalase activity appeared to be media dependent. An example of this effect is shown in Fig. 5. Catalase activity in both planktonic and biofilm cells of the *rhlI* mutant grown in TSB medium was near wild-type levels (Fig. 5A) and is in contrast to that observed with this mutant in experiments using L broth (compare with Fig. 1B). Regardless of medium, however, catalase activity in biofilms of each strain was consistently only about 10–20% of that recorded in their respective planktonic culture counterparts, showing the same relative differences for each strain (Fig. 5A).

After exposure of planktonic cultures of each strain to 75 mM $H_2O_2$ for 30 min, the log reduction in viable cells ranged between 0.73 and 2.5 (Fig. 5B). The PAI mutants were more susceptible than wild-type bacteria in each case ($P < 0.004$). Similarly, PAI mutants in biofilms were also more susceptible to $H_2O_2$ than the wild-type strain ($P < 0.04$). Each strain exhibited significantly less killing ($P < 0.06$) in biofilms relative to planktonic cultures, even though the duration of $H_2O_2$ exposure in the biofilms was four times longer than that of planktonic cells. Generally,

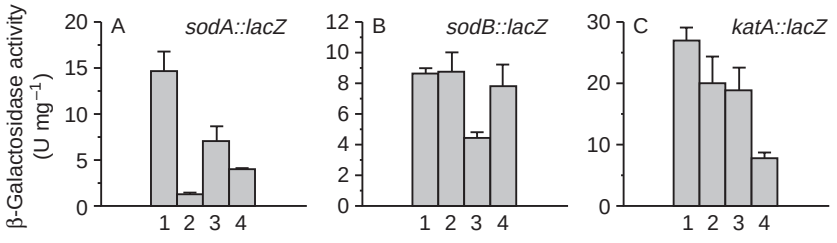


**Fig. 4.** Effect of *P. aeruginosa* autoinducers on transcription of *sodA* (A), *sodB* (B) and *katA* (C). Wild-type and autoinducer mutant organisms harbouring *lacZ* fusion plasmids pDJH201 (*sodA*::*lacZ*), pJFM202 (*sodB*::*lacZ*) and pJFM203 (*katA*::*lacZ*) were grown aerobically to stationary phase in the presence of appropriate antibiotics. β-Galactosidase activity was assayed in cell-free extracts and expressed as IU $mg^{-1}$ ($n = 3$). Lane 1, PAO1; lane 2, PAO-JP1 (*lasI*); lane 3, PDO100 (*rhlI*); lane 4, PAO-JP2 (*lasIrhlI*).

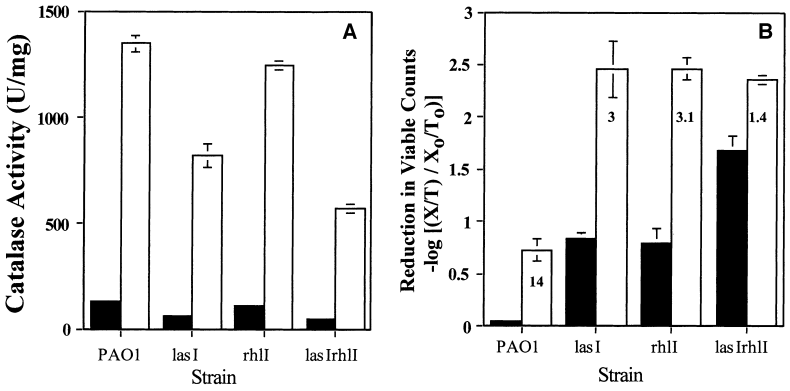


**Fig. 5.** Catalase activity and sensitivity to $H_2O_2$ of *P. aeruginosa* wild-type and autoinducer mutants in planktonic and biofilm culture.
A. Catalase specific activity in extracts of planktonic and biofilm cells.
B. $Log_{10}$ reduction in viable counts of cells sampled after exposure to $H_2O_2$. Planktonic cells, open bars; biofilms, solid bars. Both planktonic cultures and biofilms were grown in TSB and exposed to 75 mM $H_2O_2$ as described in *Experimental procedures*. In (B), the number shown in the open bars is the protective ratio derived from the ratio of viable bacteria in biofilm versus planktonic cultures after exposure to 75 mM $H_2O_2$.

$H_2O_2$ sensitivity of planktonic or biofilm bacteria appeared to be catalase dependent, as the least killing was associated with the greatest catalase activity (Fig. 5). However, a notable exception to this trend was the *rhlI* mutant, which, in both planktonic and biofilm cells, showed nearly wild-type catalase activity, yet was significantly more sensitive to $H_2O_2$ than wild-type bacteria. Also of interest, in planktonic cultures, both single mutants were as sensitive to $H_2O_2$ as the double mutant, whereas the effects of both *lasI* and *rhlI* mutations appeared nearly additive in biofilms (Fig. 5B).

Finally, the killing efficiency of biofilm and planktonic organisms was compared using the ratio of planktonic/biofilm viable cells as an indicator of the protection against $H_2O_2$ afforded to biofilm cells. Wild-type biofilms were 14-fold more resistant than wild-type planktonic cells, while resistance of the single and double PAI mutants was significantly attenuated (ratios of 3 and 3.1). Interestingly, the double mutant killing ratio of 1.4 indicates that $H_2O_2$ kills biofilm and planktonic *lasIrhlI* mutants nearly as efficiently (Fig. 5B).

## Discussion

QS in Gram-negative bacteria was postulated and proved nearly 30 years ago by Hastings and colleagues in the fish symbiont *Vibrio fischeri* (Nealson *et al*., 1970). Since then, QS has drawn intense interest in medically and agriculturally important Gram-negative genera, including *Pseudomonas*, *Vibrio*, *Rhizobium*, *Agrobacterium*, *Yersinia* and *Salmonella* (for review, see Greenberg, 1997 and website www.nottingham.ac.uk/quorum for more organisms), as well as in several Gram-positive organisms (Kleerebezem *et al*., 1997). In *P. aeruginosa*, the *las–rhl* QS system has been shown to activate the expression of numerous genes involved in mammalian virulence and stationary phase survival (Latifi *et al*., 1996; Pesci *et al*., 1997) and is controlled by the global regulators Vfr (Albus *et al*., 1997) and GacA (Reimmann *et al*., 1997). Our observations showing QS involvement in *sodA*, *sodB* and *katA* expression are entirely consistent with earlier studies, which demonstrated that SOD and catalase isozyme activities are maximal in stationary phase where QS effects are also maximal (Brown *et al*., 1995; Hassett *et al*., 1992; 1993; 1995; Ma *et al*., 1999). Thus, this study adds significantly to our understanding of how *P. aeruginosa* regulates its oxidative stress response(s) in both planktonic and biofilm organisms, and also adds to the growing list of *P. aeruginosa* cellular functions controlled by QS. A model of QS regulatory circuitry, updated from that described by Pesci *et al*. (1997), is offered in Fig. 6, and each oxidative stress function is discussed separately below.

### *Mn-SOD*

As measured at both the biochemical (Figs 1B and 2B) and gene transcriptional/translational (Fig. 4A) levels, PAI-1 was found to be essential for optimal transcription of the *fagA–fumC–orfX–sodA* operon. Transcriptional repression of this operon is controlled by the global regulator Fur (ferric uptake regulator; Prince *et al*., 1992; Hassett *et al*., 1996; 1997a,b), which uses iron as a co-repressor. Thus, when *P. aeruginosa* is exposed to an iron chelator or possesses *fur* mutations (reflecting an iron-starved status; Prince *et al*., 1992), elevated Mn-SOD activity is observed (Hassett *et al*., 1996; 1997a,b). An examination of the *fagA* promoter region revealed two Fur binding sites (i.e. iron control; Hassett *et al*., 1996; 1997a) and two putative Lux boxes (i.e. QS control; data not shown). While not yet verified experimentally, the putative Lux boxes would be candidate binding sites for the LasR–PAI-1–RNA polymerase complex and are consistent with the demonstrated influences of autoinducers. Thus, it appears that, for *sodA*, the QS regulatory system is integrated with iron-sensitive circuitry that activates transcription of the operon regardless of growth phase (Hassett *et al*., 1996). We note that this is also consistent with the effects of iron availability on production of the *P. aeruginosa*

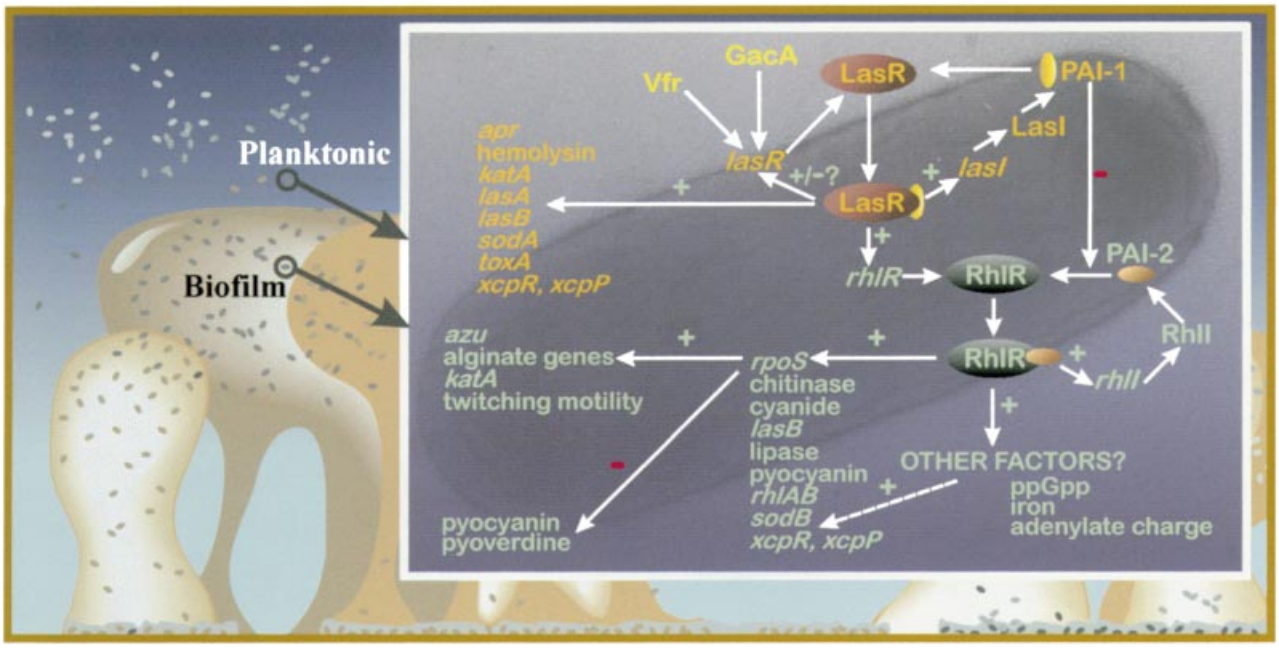


**Fig. 6.** Model of QS control in *P. aeruginosa*. This figure is based upon many research contributions within the past 6 years concerning genes and gene products under quorum-sensing control in *P. aeruginosa*. It provides an updated version of a previous tier of control reported by Pesci *et al*. (1997). The following genes and gene products are known to be influenced by QS. Many of the relevant references are given. A plus (+) sign indicates positive regulation, while a minus (−) sign indicates negative regulation. The ± ? sign indicates that LasR and Vfr may regulate one another (S.E.H. West, unpublished observations).
Upper tier regulation. The global regulators VfrA and GacA control the *las* system (Albus *et al*., 1997; Reimmann *et al*., 1997).
Genes or proteins under control of LasR: the second tier (alphabetical). *apr*, encoding alkaline protease (Gambello *et al*., 1993); *fagA–fumC–orfX–sodA* operon, with the final gene encoding Mn-SOD (this study); haemolysin (Latifi *et al*., 1996); *katA*, encoding KatA (this study); *lasA*, encoding a staphylolytic protease (Latifi *et al*., 1996); *lasB*, encoding elastase (Passador *et al*., 1993); *lasI*, encoding LasI, a protein that catalyses the synthesis of PAI-1 from acyl–acyl carrier proteins and S-adenosylmethionine (Pesci *et al*., 1997); *rhlR* encoding RhlR, the second QS transactivator (Ochsner and Reiser, 1995); twitching motility (Glessner *et al*., 1999; Suh *et al*., 1999); *toxA*, encoding exotoxin A (Gambello *et al*., 1993); *xcpP–xcpR* (Chapon-Herve *et al*., 1997).
Genes or proteins under the control of RhlR: the third tier (alphabetical). Chitinase (Winson *et al*., 1995); *katA* (this study); *lasB* (Brint and Ohman, 1995); lipase (Reimmann *et al*., 1997); pyocyanin (Brint and Ohman, 1995 , p. 1402); *rhlAB*, encoding a rhamnosyltransferase involved in the synthesis of the rhamnolipid surfactant; *rhlI*, encoding RhlI, a protein that catalyses the synthesis of PAI-2 from acyl–acyl carrier proteins and S-adenosylmethionine (Pesci *et al*., 1997); *sodB*, encoding Fe-SOD (potentially indirect control as indicated by 'other factors?' in figure, this study); *xcpP-xcpR* (Chapon-Herve *et al*., 1997).
Genes or compounds under the control of *rpoS* (alphabetical). Genes involved in alginate production on solid medium (Suh *et al*., 1999); *azu*, encoding an azurin (Vijgenboom *et al*., 1997); *katA* (Suh *et al*., 1999); genes involved in pyocyanin and pyoverdine biosynthesis (Suh *et al*., 1999).

virulence factors elastase, exotoxin A and pyoverdine (Sokol *et al*., 1982).

### *Fe-SOD*

In contrast to what appears to be a requirement of PAI-1 for control of the *fagA–fumC–orfX–sodA* operon under normal growth conditions (i.e. no significant iron deprivation), control of *sodB* transcription appears to be unaffected by PAI-1 and is only partially mediated by PAI-2 (Figs 2B and 4B). The *rhlI* mutant produced ≈ 50% of the Fe-SOD activity found in the wild-type strain PAO1 and is in agreement with previous reports, which showed that Fe-SOD activity is detectable under all growth conditions, but is at maximum levels in stationary phase planktonic cells (Hassett *et al*., 1992; 1995). Unlike Mn-SOD activity, however, Fe-SOD activity is maximal when iron is plentiful (Hassett *et al*., 1992).

### *KatA*

Both PAI-1 and PAI-2 appear to play a role in regulating *katA*, but not to the apparent 'all-or-none' level seen with *sodA*. Similar to that found in the promoter region upstream of the *fagA–fumC–orfX–sodA* operon, DNA sequence upstream of *katA* contains candidate Lux boxes (Ma *et al*., 1999; GenBank no. AF047025). KatA is the primary catalase produced by *P. aeruginosa* (Hassett *et al*., 1992; Brown *et al*., 1995), and thus constitutes the first line of defence against $H_2O_2$. Upon exposure to $H_2O_2$, *P. aeruginosa* activates transcription of *katA* (Ma *et al*., 1999) and, to a much greater extent, *katB* (Brown *et al*.,

1995). In both planktonic and biofilm cells, mutants lacking one or both autoinducers possessed less KatA activity and increased sensitivity to $H_2O_2$ relative to wild-type cells (Figs 1A, 1B, 2A and 5). Consistent with these findings, Suh *et al*. (1999) have recently shown decreased catalase activity and increased sensitivity to $H_2O_2$ in *rpoS* mutants, with *rpoS* being under the control of the *rhl* QS system. Furthermore, an isogenic *katA* mutant was killed much more efficiently than wild-type or *katB* strains in both planktonic and biofilm cultures (Elkins *et al*., 1999). The *katA*::*lacZ* reporter data (Fig. 4C) was in agreement with the enzyme activity data, although the results were less resolute in the *lasI* and *rhlI* single mutants. Also, like the SODs, KatA expression is sensitive to iron availability (Hassett *et al*., 1992; 1993; 1996; 1997a,b). Because most catalases contain haem, catalase activity is predictably greatest when organisms are grown in iron-rich media (Hassett *et al*., 1992; Ma *et al*., 1999). Thus, again, it appears that QS circuitry in *P. aeruginosa* is integrated with the iron status of the cell and, therefore, in the context of cell nutrition is similar to a phenomenon observed recently in the carbon starvation response in both *P. aeruginosa* (Vandelden *et al*., 1998) and *Vibrio* sp. (Srinivasan *et al*., 1998). Indeed, carbon-starved *P. aeruginosa lasI* mutants could still produce elastase and rhamnolipid, a phenomenon that was presumed to result from elevated levels of compensatory RhlR. This is not surprising given that PAIs accumulate to activating levels when cell densities are high, a situation that creates a high demand for critical nutrients such as iron that have limited solubility under aerobic conditions. To summarize, the results observed from our studies with *katA* as well as *sodB* suggest that autoinducers may only play a modulating role in the expression of some genes. Also, there may be different versions of autoinducers, which will effect transcription to differing degrees depending on their affinity for their cognate regulatory protein(s). It is also possible that similar but different autoinducer molecules result in varying conformations of the regulatory protein–autoinducer–RNA polymerase complex that, in turn, will vary with respect to DNA-binding properties (see below).

### *QS regulatory complexities*

During this study, we became puzzled as to why a single QS mutant would display a mutant phenotype (e.g. decreased Fe-SOD activity of the *rhlI* mutant), while the double mutant possessed wild-type levels (Figs 2B and 4B). This may represent the second example of negative autoinducer interaction within the Las–Rhl regulatory system, a phenomenon reported recently by Pesci *et al*. (1997). PAI-1 was shown to inhibit binding of PAI-2 to RhlR, resulting in the significantly reduced expression of *rhlA* (Pesci *et al*., 1997), a gene controlled by the RhlR–RhlI tandem (Ochsner and Reiser, 1995). Binding of PAI-1 to RhlR may be competitive with respect to PAI-2, rendering RhlR less capable of binding to its target promoter sequences. In the experiments reported here, the binding of PAI-1 to RhlR in the *rhlI* mutant could result in the inhibition of RhlR activation of *sodB*. However, in the *lasIrhlI* double mutant, synthesis of this putative antagonist would be eliminated and thus allow RhlR to interact with targeted genes, resulting in the near wild-type levels of gene expression in the double mutant (Figs 2B and 4B). Based upon current theory regarding the requirement of autoinducers for efficient binding of regulatory proteins (e.g. LasR or RhlR) to target DNA sequences, this scenario would require a replacement for PAI-2. Although quantitatively less prevalent than PAI-2, the occurrence of a PAI-2 structural analogue that functionally replaces PAI-2 has also been found in culture filtrates of *P. aeruginosa* grown in L broth (Winson *et al*., 1995). Therefore, in the experiments with the *lasIrhlI* mutant, it is possible that this alternative autoinducer allowed for near-optimum RhlR-directed gene transcription in the absence of PAI-1 synthesis.

Another interesting observation encountered during the course of this study was the growth medium-dependent variation of KatA catalase levels in the *rhlI* mutant. When cultured in L broth, catalase levels in this strain were significantly less than in the wild-type strain and similar to that found in the *lasI* mutant (Fig. 1B). However, when this mutant was grown in 1:10 TSB medium, *rhlI* mutant catalase activity was consistently near that of the wild-type strain (Fig. 5A and three additional independent experiments, results not shown). This increase in catalase specific activity was not caused by induction of *katB*, as there was no KatB activity detected (results not shown), and was consistent in experiments with both planktonic cells and biofilms (Fig. 5). Similar to the explanation offered above for mutant-dependent Fe-SOD levels, it is perhaps possible that a PAI-2 alternative may serve the role of transcriptional effector, with its synthesis varying under different growth/media conditions. The absence of a growth medium influence on KatA levels in either the *lasI* mutant or *lasIrhlI* double mutant, however, suggests that the Las portion of the cascade is indispensable.

It is also possible that LasR and RhlR may possess different capacities to activate *las*- and *rhl*-controlled genes, depending on the presence and concentration of autoinducer. We base the second hypothesis on two recent studies in the phytopathogenic bacterium *Erwinia chrysanthemi* (Nasser *et al*., 1998; Reverchon *et al*., 1998). It was found that the LasR homologue, ExpR, bound to different DNA sequences of promoter regions of genes under ExpR control depending upon the presence, absence and concentration of *N*-(3-oxohexanoyl)- homoserine lactone. We postulate that *P. aeruginosa* LasR and RhlR could act in

a similar fashion and activate some *las*- or *rhl*-controlled genes differently under such conditions.

### *Quorum sensing in biofilm versus planktonic culture*

When challenged with $H_2O_2$, all biofilm bacteria in this study displayed the reduced susceptibility to disinfection that is a hallmark of the protective nature of the biofilm microniche (Costerton *et al*., 1995; Elkins *et al*., 1999; Hassett *et al*., 1999). Part of this is explained by the varying levels of catalase, an important defence against $H_2O_2$. This conclusion is supported by a recent report by Elkins *et al*. (1999), who demonstrated that biofilms formed by a *P. aeruginosa katA* mutant are easily killed by $H_2O_2$. However, the results of the $H_2O_2$ exposure experiments in the present study also revealed that there are other important $H_2O_2$ resistance mechanisms that may be unique to biofilm cells and controlled by QS. In the wild-type strain, the degree of protection afforded by the biofilm mode of growth exceeded planktonic cells by a factor of 14. However, the protection factor calculated for the lasI and rhlI mutants was $\approx 3$, and that of the *lasIrhlI* mutant was only 1.4 (Fig. 5B). In addition, although the *rhlI* mutant exhibited near wild-type catalase activity regardless of growth mode, it was much more sensitive to $H_2O_2$ in both cell types. In combination with the fact that catalase activity in biofilms was only a fraction of that observed with planktonic cells (Fig. 5A), these observations demonstrate that QS signalling mediates a significant component of biofilm resistance to $H_2O_2$, and that the additional $H_2O_2$ resistance found in biofilm organisms cannot, at this point, be completely attributed to differences in catalase activity. These putative QS-mediated mechanism(s) remain to be identified, but could perhaps include elements of cell nutrition, such as iron availability, adequate phosphorus to facilitate normal phosphorelay for pertinent two-component regulatory protein pairs found to be involved in QS control, or carbon/energy source availability (Ostling *et al*., 1996; Srinivasan *et al*., 1998; Vandelden *et al*., 1998). Each could influence energy metabolism (e.g. adenylate charge) or involve elements of the stringent response (e.g. ppGpp levels, see Fig. 6) (Huisman and Kolter, 1994; Ostling *et al*., 1996; Srinivasan *et al*., 1998). It is anticipated that future work will begin to identify those regulatory elements that are integrated with QS circuitry.

### *Summary*

In less than 6 years, increasing numbers of genes under QS control have been found in *P. aeruginosa* (Fig. 6). Some of these genes are known to be involved in animal virulence (elastase, exotoxin A, rhamnolipid and pyoverdine), and others are involved in the architecture, SD and $H_2O_2$ resistance of biofilm-grown cells (Davies *et al*., 1998; this study). Because of the clinical and environmental problems caused by *P. aeruginosa*, we predict the development of both PAI structural analogues that bind and inactivate LasR or RhlR, and competitive inhibitors of the LasI and RhlI autoinducer synthase enzymes. Observations made in this study, and also those reported previously (Pesci *et al*., 1997), provide evidence that negative autoinducer interactions occur and suggest that analogue and/or autoinducer synthase inhibitor therapy may be important future tools in the control of diseases caused by quorum-sensing organisms. However, *P. aeruginosa* synthesizes additional autoinducers capable of replacing PAI-1 or PAI-2 and, thus, analogue design will perhaps have to account for the occurrence of multiple activator molecules. Clinically, if such compounds do not elicit a potentially harmful immunomodulatory response (e.g. PAI-1; Telford *et al*., 1998), they could be used as novel therapeutic agents, either alone or in tandem with current antibiotic regimens. Implications for the treatment of *P. aeruginosa* biofilm infections, such as those involving colonization of catheters and the pulmonary airways of cystic fibrosis patients, could be significant. Also of potential significance are the observations that the nutritional status of the cell can modulate signalling-regulated responses (this study; Srinivasan *et al*., 1998; Vandelden *et al*., 1998). Prior treatment of problematic biofilms with analogue compounds, or manipulation of nutrient conditions that paralyze QS circuitry, may allow for greater biofilm control or killing when followed by appropriately timed biocide application.

## Experimental procedures

### *Bacterial strains, plasmids and media*

Properties of the bacteria and plasmids used in this study are shown in Table 1. Planktonic and biofilm cultures were grown aerobically in either Luria (L) broth (10 g $l^{-1}$ tryptone, 5 g $l^{-1}$ yeast extract, 5 g $l^{-1}$ NaCl) or 1:10 trypticase soy broth (TSB; 3 g $l^{-1}$ trypticase soy, 0.5 g $l^{-1}$ yeast extract) at 37°C. Frozen stocks were stored indefinitely at −80°C in a 1:1 mixture of 25% glycerol and bacteria grown in L broth to stationary phase.

### *Construction of isogenic mutants*

The strategy for insertional inactivation of genes of interest in wild-type strain PAO1 was facilitated using the gene replacement vector pEX100T, which allowed for selection of double cross-over events in the presence of 6% sucrose (Schweizer and Hoang, 1995). Genes were interrupted with either an *aaC1* cassette (Schweizer, 1993), encoding an aminoglycoside acetylase, or a cassette encoding resistance to tetracycline derived from pBR322. All mutants were confirmed by Southern analysis.

**Table** 1. Strains and plasmids used in this study.

| Strain and plasmids | Genotype or characteristics[a] | Source or reference |
|---|---|---|
| *E. coli* | | |
| DH5α | F-*lacZ*DM15 *recA1 hsdR17 supE44* Δ(*lacZYA argF*) | Bethesda Research Laboratories |
| SM10 | Km$^r$, *mob*+ *tra*+, *thi*-1 *thr leu tonA lacY supE recA*::RP4-2-Tc::Mu, mobilizer strain | Simon *et al*. (1983) |
| *P. aeruginosa* | | |
| PAO1 | Wild type, prototrophic | Holloway (1969) |
| PAO-JP1 | Tc$^{r-}$ *las*I::Tn*10,* PAO1 derivative | Pearson *et al*. (1997) |
| PDO100 | Hg$^r$, *rhlI*::Tn*501-2,* PAO1 derivative | Brint and Ohman (1995) |
| PAO-JP2 | Hg$^r$, Tc$^r$, *lasI*::Tn*10*, *rhlI*::Tn*501-2*, PAO1 derivative | Pesci *et al*. (1997) |
| *sodA* | *sodA*::Gm | Hassett *et al*. (1995) |
| *sodB* | *sodB*::Cb | Hassett *et al*. (1995) |
| *sodAsodB* | *sodA*::Gm, *sodB*::Cb | Hassett *et al*. (1995) |
| *katA* | *katA*::Gm | Ma *et al*. (1999) |
| *katB* | *katB*::Gm | This study |
| *katAkatB* | *katA*::Gm, *katB*::Tc | This study |
| Plasmids | | |
| pUC18 | Ap$^r$, Cloning vector | Yanisch-Perron *et al*. (1985) |
| pKS-(Bluescript) | Ap$^r$, Extended polylinker pUC derivative | Stratagene |
| pZ1918 | Ap$^r$, pUC19/18 with 3.2 kb *lacZ* cassette | Schweizer (1993) |
| pZ1918G | Ap$^r$, Gm$^r$, pZ1918 with Gm$^r$ cassette immediately downstream of the *lacZ* gene | H. P. Schweizer |
| pPZ30 | Ap$^r$, broad-host-range vector | Schweizer (1991) |
| pQF50 | Ap$^r$, ColE1 *ori-oriR* with multiple cloning site with promoterless *lacZ* gene | Farinha and Helinski (1990) |
| pUCP21T | Ap$^r$, broad-host-range vector | West *et al*. (1994) |
| pDJH10 | Ap$^r$, pKS-with ≈ 1.7 kb *Bam*HI–*Pst*I *sodA* fragment | Hassett *et al*. (1995) |
| pDJH7 | Ap$^r$, pKS-with ≈ 3.4 kb *Pst*I *sodB* fragment | Hassett *et al*. (1995) |
| pSMB2 | Ap$^r$, pKS-with ≈ 5.2 kb *Eco*RI fragment containing *katB*, *ankB* and *radA* | Brown *et al*. (1995) |
| pJFM200 | Ap$^r$, pKS-with ≈ 3.8 kb *Eco*RI–*Eco*RV fragment containing *rpoA*, *rpsD*, *katA* and *bfrA* | This study |
| pDJH201 | Ap$^r$, pUCP21T with 7.8 kb *fagA–fumC–orfX–sodA* with *lacZ*-Gm in *Sph*I site of *sodA* | This study |
| pJFM202 | Ap$^r$, pPZ30 with 247 bp *Eco*RI–*Pst*I *sodB* fused to promoterless *lacZ* gene | This study |
| pJFM203 | Ap$^R$, pUCP21T plus 3.8 kb *Eco*RI–*Eco*RV fragment containing *rpoA*, *rpsD*, *katA* and *bfrA* with *lacZ*-Gm cassette in *Sma*I site of *katA* | This study |
| pJE26 | Ap$^r$, *Xba*I–*Hin*dIII *katB* fragment in pQF50, forming a *katB–lacZ* transcriptional fusion | This study |

**a**. Abbreviations used for genetic markers were as described previously (Holloway *et al*., 1979). *mob*, mobilization site (ColE1); *tra*, conjugative phenotype; *oriR*, replicative origin; Ap$^r$, ampicillin resistance; Gm$^r$, gentamicin resistance; Km$^r$, kanamycin resistance; Hg$^r$, mercury resistance.

### *Sensitivity to $H_2O_2$ and phenazine methosulphate (PMS)*

Bacteria were grown aerobically at 37°C with shaking at 300 r.p.m. for 17 h. For $H_2O_2$ sensitivity experiments, suspensions (0.1 ml) were diluted in 3 ml of molten soft agar (0.6%), poured onto the surface of L agar plates, and the agar was allowed to solidify. Filter paper disks (7 mm, Whatman no. 1) saturated with 10 ml of 30% $H_2O_2$ were placed on the agar overlay. For PMS sensitivity experiments, a 0.1 ml suspension of stationary phase bacteria was spread over the entire surface of L agar plates. After 17 h of growth at 37°C, the stationary phase organisms were removed from the agar surface with 10 ml of 0.9% saline and a sterile glass rod. Filter paper disks impregnated with 1 M PMS (pH 7.0) were placed on the agar surface. Sensitivity to each agent was then recorded as the mean ± SE ($n = 9$) of the diameter of growth inhibition after 24 h of growth at 37°C.

### *PAI add-back experiments*

PAI-1 and PAI-2 were synthesized as described previously (Passador *et al*., 1996). *P. aeruginosa* strains were grown aerobically at 37°C in L broth containing 1 mM PAI-1, PAI-2 or both autoinducers. Ethyl acetate, used to solubilize PAI-1 and PAI-2, was added to control suspensions to a final concentration of 0.2% (v/v). Organisms were incubated aerobically for 17 h followed by preparation of cell-free extracts (described below).

### *Reporter gene experiments using* lacZ

Reporter plasmids were designed to monitor transcriptional or translational activity of the *sodA*, *sodB*, *katA* and *katB* loci fused to the *lacZ* gene (Table 1). Each construct was conjugated via triparental or biparental mating into PAO1, and the *lasI*, *rhlI* and *lasIrhlI* mutants. Cultures of each strain were grown to stationary phase in L broth, collected by centrifugation, washed and ruptured by sonication. β-Galactosidase activity was assayed in cell extracts using ONPG, and the results expressed as international units $mg^{-1}$ protein using an extinction coefficient for ONPG of 3.1 (Miller, 1992). Protein concentrations were estimated according to the method of Bradford (1976) using bovine serum albumin fraction V (Sigma) as standard.

### *Biofilm experiments*

Biofilms were grown using a drip flow reactor as described previously (Huang *et al*., 1998). Briefly, 1/100 TSB was dripped over sterile stainless steel coupons (316 l, 1.3 × 7.6 cm) held in parallel polycarbonate chambers. Each coupon, resting horizontally in the polycarbonate chamber, was inoculated with 1 ml of overnight culture and 15 ml of fresh 1/100

TSB. The reactor cover was closed, and bacteria were allowed to attach in a static environment for an 18 h period. The entire reactor was then inclined at 10°, and the nutrient flow (50 ml $h^{-1}$) was initiated. Medium dripped onto the coupon at the raised edge and flowed down lengthwise over the coupon and out of an effluent port at the chamber base. Depending on the strain, biofilms were grown for 24–48 h at 25°C.

### *Planktonic versus biofilm $H_2O_2$ susceptibility*

Planktonic bacteria were grown overnight at 25°C in TSB and diluted 1:100 in the same medium containing 75 mM $H_2O_2$. After 30 min, sodium thiosulphate (0.2% w/v) was added to neutralize the $H_2O_2$. The suspension was serially diluted, and aliquots were plated on R2A agar (a medium optimal for rescuing damaged cells; Reasoner and Geldreich, 1985). Colony-forming units were enumerated after a 24 h incubation at 37°C, with the log reduction in viability calculated as the comparison of final versus initial viable cell counts of cells taken just before $H_2O_2$ addition. Biofilms were treated by continuous flow of 1/10 TSB containing 75 mM $H_2O_2$ at 50 ml $h^{-1}$ for 2 h. After treatment, coupons were scraped into 50 ml of phosphate buffer containing 0.2% sodium thiosulphate and homogenized using a Brinkman homogenizer (model PT 10/35). Homogenized biofilms were analysed for viable bacteria by serial dilution and plating as for planktonic cultures. The resuspended biofilms were also analysed for total cell numbers by acridine orange direct counts. The same assays were performed on untreated control biofilm coupons to verify the reliability of comparisons between total and viable counts, and for direct comparison against $H_2O_2$-treated cultures or biofilms. Log reduction of viable bacteria in biofilms was calculated based on the initial and final surviving fractions (defined as the ratio of colony-forming units to direct microscopic counts). This approach factors out the detachment of cells occurring in biofilm experiments that is not a true measurement of disinfection. All experiments were conducted at least three times.

### *Cell extract preparation, non-denaturing gel electrophoresis and biochemical assays*

Cell extracts were prepared from bacteria harvested by centrifugation at 10 000 × *g* for 10 min at 4°C. Organisms were washed twice in ice-cold 50 mM potassium phosphate buffer, pH 7.0, and sonicated in an ice water bath for 10 s with a Heat-Systems model W-225 sonicator at setting 5. The sonicate was clarified by centrifugation at 13 000 × *g* for 10 min at 4°C. Catalase activity was monitored by following the decomposition of 18 mM $H_2O_2$ in 50 mM potassium phosphate buffer, pH 7.0, at 240 nm (Beers and Sizer, 1952; Brown *et al*., 1995; Hassett *et al*., 1996). One unit of activity was that which decomposes 1 mmol of $H_2O_2$ $min^{-1}$ $mg^{-1}$. SOD activity was monitored by the SOD-inhibitable autoxidation of pyrogallol (Marklund and Marklund, 1974) with modifications specified by Steinman (1985). An aliquot (≈ 5 ml) of a freshly prepared 10 mM stock of pyrogallol in 10 mM HCl was added to oxygenated 50 mM Tris-HCl/1 mM EDTA, pH 8.2, and mixed thoroughly. The change in absorbance at 320 nm was recorded for 1 min. The volume of pyrogallol added was adjusted until the change in $OD_{320}$ was 0.02 ± 0.002. The amount of cell extract that caused a 50% reduction in pyrogallol autooxidation (e.g. $DOD_{320} = 0.01 \pm 0.001$) constituted 1 U of activity. Specific activity was then calculated as U $mg^{-1}$ protein. Cell extracts for native gel electrophoresis were prepared as above, but in 50 mM Tris-HCl, pH 7.4. Catalase activity staining of 6% non-denaturing polyacrylamide gels was performed according to the method of Wayne and Diaz (1986). Briefly, the gels were soaked in distilled water for 5 min, followed by a 10 min incubation in 10 mM $H_2O_2$ at room temperature. The $H_2O_2$ solution was replaced by distilled water and incubated for an additional 5 min. The distilled water was replaced by a solution of 1% ferric chloride–potassium ferricyanide, and the insoluble Prussian blue pigment was allowed to develop until achromatic catalase activity bands were clearly visible. The molecular basis for this stain is as follows. $H_2O_2$ reduces potassium ferricyanide to potassium ferrocyanide, which, in turn, reacts with ferric chloride to form Prussian blue (or Berlin blue plus hexacyanoferrate). Gels were finally rinsed in distilled water and photographed. SOD activity gel staining of 10% non-denaturing polyacrylamide gels was accomplished according to the method of Clare *et al*. (1984).

### *Statistics*

Where applicable, Student's *t*-test was used to determine the statistical significance of differences between treatments.

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## References

Albus, A.M., Pesci, E.C., Runyen-Janecky, L., West, S.E.H., and Iglewski, B.H. (1997) Vfr controls quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol* **179:** 3928–3935.

Beers, Jr, R.F., and Sizer, I.W. (1952) A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J Biol Chem* **195:** 133–140.

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.

Brint, J.M., and Ohman, D.E. (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.

Brown, S.M., Howell, M.L., Vasil, M.L., Anderson, A.J., and Hassett, D.J. (1995) Cloning and characterization of the

*katB* gene of *Pseudomonas aeruginosa* encoding a hydrogen peroxide-inducible catalase: purification of KatB, cellular localization, and demonstration that it is essential for optimal resistance to hydrogen peroxide. *J Bacteriol* **177:** 6536–6544.

Chapon-Herve, V., Akrim, M., Latifi, A., Williams, P., Lazdunski, A., and Bally, M. (1997) Regulation of the *xcp* secretion pathway by multiple quorum-sensing modulons in *Pseudomonas aeruginosa*. *Mol Microbiol* **24:** 1169–1178.

Clare, D.A., Duong, M.N., Darr, D., Archibald, F., and Fridovich, I. (1984) Effects of molecular oxygen on the detection of superoxide radical with nitroblue tetrazolium and an activity stain for catalase. *Anal Biochem* **140:** 532–537.

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korbe, D.R., and Lappin-Scott, H.M. (1995) Microbial biofilms. *Annu Rev Microbiol* **49:** 711–745.

Costerton, J.W., Lewandowski, Z., DeBeer, D., Caldwell, D., Korber, D., and James, G. (1994) Biofilms, the customized microniche. *J Bacteriol* **176:** 2137–2142.

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

Elkins, J.G., Hassett, D.J., Stewart, P.S., Schweizer, H.P., and McDermott, T.R. (1999) Protective role of catalase in *Pseudomonas aeruginosa* biofilm resistance to hydrogen peroxide. *Appl Environ Microbiol* **65:** 4594–4600.

Farinha, M.A., and Helinski, A.M. (1990) Construction of broad-host-range plasmid vectors for easy visible selection and analysis of promoters. *J Bacteriol* **172:** 3496–3499.

Fuqua, C., Winans, S.C., and Greenberg, E.P. (1996) Census and consensus in bacterial ecosystems: the LuxR–LuxI family of quorum-sensing transcriptional regulators. *Annu Rev Microbiol* **50:** 727–751.

Gambello, M.J., Kaye, S., and Iglewski, B.H. (1993) LasR of *Pseudomonas aeruginosa* is a transcriptional activator of the alkaline protease gene (*apr*) and an enhancer of exotoxin A expression. *Infect Immun* **61:** 1180–1184.

Glessner, A., Smith, R.S., Iglewski, B.H., and Robinson, J.B. (1999) Roles of *Pseudomonas aeruginosa las* and *rhl* quorum-sensing systems in control of twitching motility. *J Bacteriol* **181:** 1623–1629.

Govan, J.R.W., and Harris, G.S. (1986) *Pseudomonas aeruginosa* and cystic fibrosis: unusual bacterial adaptation and pathogenesis. *Microbiol Sci* **3:** 302–308.

Greenberg, E.P. (1997) Quorum sensing in Gram-negative bacteria. *Am Soc Microbiol News* **63:** 371–377.

Hassett, D.J., Charniga, L., Bean, K.A., Ohman, D.E., and Cohen, M.S. (1992) Antioxidant defense mechanisms in *Pseudomonas aeruginosa:* resistance to the redox-active anti-biotic pyocyanin and demonstration of a manganese-cofactored superoxide dismutase. *Infect Immun* **60:** 328–336.

Hassett, D.J., Woodruff, W.A., Wozniak, D.J., Vasil, M.L., Cohen, M.S., and Ohman, D.E. (1993) Cloning of the *sodA* and *sodB* genes encoding manganese and iron superoxide dismutase in *Pseudomonas aeruginosa*: demonstration of increased manganese superoxide dismutase activity in alginate-producing bacteria. *J Bacteriol* **175:** 7658–7665.

Hassett, D.J., Schweizer, H.P., and Ohman, D.E. (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.

Hassett, D.J., Sokol, P., Howell, M.L., Ma, J.-F., Schweizer, H.P., Ochsner, U.A., and Vasil, M.L. (1996) Ferric uptake regulator (Fur) mutants of *Pseudomonas aeruginosa* demonstrate defective siderophore-mediated iron uptake and altered aerobic metabolism. *J Bacteriol* **178:** 3996–4003.

Hassett, D.J., Howell, M.L., Sokol, P.A., Vasil, M.L., and Dean, G.E. (1997a) 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 native FumC. *J Bacteriol* **179:** 1442–1451.

Hassett, D.J., Howell, M.L., Ochsner, U.A., Johnson, Z., Vasil, M.L., and Dean, G.E. (1997b) 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.

Hassett, D.J., Elkins, J.G., Ma, J.-F., and McDermott, T.R. (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.

Holloway, B.W. (1969) Genetics of *Pseudomonas*. *Bacteriol Rev* **33:** 419–443.

Holloway, B.W., Krishnapillai, V., and Morgan, A.F. (1979) Chromosomal genetics of *Pseudomonas*. *Microbiol Rev* **43:** 73–102.

Huang, C.-T., Xu, K.D., McFeeters, G.A., and Stewart, P.S. (1998) Spatial patterns of alkaline phosphatase expression within bacterial colonies and biofilms in response to phosphate starvation. *Appl Environ Microbiol* **64:** 1526–1531.

Huisman, G.W., and Kolter, R. (1994) Sensing starvation: a homoserine lactone-dependent signaling pathway in *Escherichia coli*. *Science* **265:** 537–539.

Jiang, Y., Camara, M., Chhabra, S.R., Hardie, K.R., Bycroft, B.W., Lazdunski, A., *et al.* (1998) *In vitro* biosynthesis of the *Pseudomonas aeruginosa* quorum-sensing signal molecule *N*-butanoyl-L-homoserine lactone. *Mol Microbiol* **28:** 193–203.

Kleerebezem, M., Quadri, L.E., Kuipers, O.P., and de Vos, W.M. (1997) Quorum sensing by peptide pheromones and two-component signal-transduction systems in Gram-positive bacteria. *Mol Microbiol* **24:** 895–904.

Latifi, A., Foglino, M., Tanaka, K., Williams, P., and Lazdunski, A. (1996) A hierarchical 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.

Ma, J.-F., Ochsner, U.A., Klotz, M.G., Nanayakkara, V.K., Howell, M.L., Johnson, Z., *et al.* (1999) Bacterioferritin A modulates catalase A (KatA) activity and resistance to hydrogen peroxide in *Pseudomonas aeruginosa*. *J Bacteriol* **181:** 3730–3742.

Marklund, S., and Marklund, G. (1974) Involvement of the superoxide anion radical in the antoxidation of pyrogallol

and a convenient assay for superoxide dismutase. *Eur J Biochem* **47:** 469–474.

Miller, J.H. (1992) Procedures for working with *lac*. In *A Short Course in Bacterial Genetics*. Miller, J.H. (ed.). Plainview, NY: Cold Spring Harbor Laboratory Press, pp. 72–74.

Nasser, W., Bouillant, M.L., Salmond, G., and Reverchon, S. (1998) Characterization of the *Erwinia chrysanthemi expI-expR* locus directing the synthesis of two *N*-acyl-homoserine lactone signal molecules. *Mol Microbiol* **29:** 1391–1405.

Nealson, K.H., Platt, T., and Hastings, J.W. (1970) Cellular control of the synthesis and activity of the bacterial luminescent system. *J Bacteriol* **104:** 313–322.

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

Ostling, J.L., Holmquist, L., and Kjelleberg, S. (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.

Passador, L., Cook, J.M., Gambello, M.J., Rust, L., and Iglewski, B.H. (1993) Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. *Science* **260:** 1127–1130.

Passador, L., Tucker, K.D., Guertin, K.R., Journet, M.P., Kende, A.S., and Iglewski, B.H. (1996) Functional analysis of the *Pseudomonas aeruginosa* autoinducer PAI. *J Bacteriol* **178:** 5995–6000.

Pearson, J.P., Gray, K.M., Passador, L., Tucker, K.D., Eberhard, A., Iglewski, B.H., *et al*. (1994) Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. *Proc Natl Acad Sci USA* **91:** 197–201.

Pearson, J.P., Passador, L., Iglewski, B.H., and Greenberg, E.P. (1995) A second N-acylhomoserine lactone produced by *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **92:** 1490–1494.

Pearson, J.P., Pesci, E.C., and Iglewski, B.H. (1997) Roles of *Pseudomonas aeruginosa las* and *rhl* quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. *J Bacteriol* **179:** 5756–5767.

Pesci, E.C., Pearson, J.P., Seed, P.C., and Iglewski, B.H. (1997) Regulation of *las* and *rhl* quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol* **179:** 3127–3132.

Prince, R.W., Cox, C.D., and Vasil, M.L. (1992) Molecular cloning and sequencing of the *Pseudomonas aeruginosa fur* gene: coordinate regulation of siderophore and exotoxin A production. *J Bacteriol* **175:** 2589–2598.

Reasoner, D.J., and Geldreich, E.E. (1985) A new medium for the enumeration and subculture of bacteria from potable water. *Appl Environ Microbiol* **49:** 1–7.

Reimmann, C., Beyeler, M., Latifi, A., Winteler, H., Foglino, M., Lazdunski, A., *et al*. (1997) The global activator GacA of *Pseudomonas aeruginosa* PAO positively controls the production of the autoinducer *N*-butyryl-homoserine lactone and the formation of the virulence factors pyocyanin, cyanide, and lipase. *Mol Microbiol* **24:** 309–319.

Reverchon, S., Bouillant, M.L., Salmond, G., and Nasser, W. (1998) Integration of the quorum-sensing system in the regulatory networks controlling virulence factor synthesis in *Erwinia chrysanthemi*. *Mol Microbiol* **29:** 1407–1418.

Schweizer, H.P. (1991) Improved broad-host-range *lac*-based plasmid vectors for the isolation and characterization of protein fusions in *Pseudomonas aeruginosa*. *Gene* **103:** 87–92.

Schweizer, H.P. (1993) Two plasmids, X1918 and Z1918, for easy recovery of the *xylE* and *lacZ* reporter genes. *Gene* **134:** 89–91.

Schweizer, H.P., and Hoang, T.T. (1995) An improved system for gene replacement and *xylE* fusion analysis in *Pseudomonas aeruginosa*. *Gene* **158:** 15–22.

Simon, R., Priefer, U., and Puehller, A. (1983) A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. *Bio-Technology* **1:** 784–791.

Sokol, P.A., Cox, C.D., and Iglewski, B.H. (1982) *Pseudomonas aeruginosa* mutants altered in their sensitivity to the effect of iron on toxin A or elastase yields. *J Bacteriol* **151:** 783–787.

Srinivasan, S., Ostling, J., Charlton, T., de Nys, R., Takayama, K., and Kjellberg, S. (1998) Extracellular signal molecule (s) involved in the carbon starvation response of marine *Vibrio* sp. strain S14. *J Bacteriol* **180:** 201–209.

Steinman, H.M. (1985) Bacteriocuprein superoxide dismutases in pseudomonads. *J Bacteriol* **162:** 1255–1260.

Suh, S.J., Silo-Suh, L., Woods, D.E., Hassett, D.J., West, S.E.H., and Ohman, D.E. (1999) Effect of *rpoS* mutation on the stress response and expression of virulence factors in *Pseudomonas aeruginosa*. *J Bacteriol* **181:** 3890–3897.

Telford, G., Wheeler, D., Williams, P., Tomkins, P.T., Appleby, P., Sewell, H., *et al*. (1998) The *Pseudomonas aeruginosa* quorum-sensing molecule N-(3-oxododecanoyl)-L-homoserine lactone has immunomodulatory activity. *Infect Immun* **66:** 36–42.

Vandelden, C., Pesci, E.C., Pearson, J.P., and Iglewski, B.H. (1998) Starvation selection restores elastase and rhamnolipid production in a *Pseudomonas aeruginosa* quorum-sensing mutant. *Infect Immun* **66:** 4499–4502.

Vijgenboom, E., Busch, J.E., and Canters, G.W. (1997) *In vivo* studies disprove an obligatory role of azurin in denitrification in *Pseudomonas aeruginosa* and show that *azu* expression is under control of *rpoS* and ANR. *Microbiology* **143:** 2853–2863.

Wayne, L.G., and Diaz, G.A. (1986) A double staining method for differentiating between two classes of mycobacterial catalase in polyacrylamide gels. *Anal Biochem* **157:** 89–92.

West, S.E.H., Schweizer, H.P., Dall, C., Sample, A.K., and Runyen-Janecky, L.J. (1994) Construction of improved *Escherichia–Pseudomonas* shuttle vectors derived from pUC18/19 and sequence of the region required for their replication in *Pseudomonas aeruginosa*. *Gene* **148:** 81–86.

Winson, M.K., Camara, M., Latifi, A., Foglino, M., Chhabra, S.R., Daykin, M., *et al*. (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.

Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Improved M13 cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33:** 103–119.