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Ferric Uptake Regulator (Fur) Mutants of *Pseudomonas aeruginosa* Demonstrate Defective Siderophore-Mediated Iron Uptake, Altered Aerobic Growth, and Decreased Superoxide Dismutase and Catalase Activities

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Pseudomonas aeruginosa **is considered a strict aerobe that possesses several enzymes important in the disposal of toxic oxygen reduction products including iron- and manganese-cofactored superoxide dismutase and catalase. At present, the nature of the regulation of these enzymes in *P. aeruginosa* is not understood. To address these issues, we used two mutants called A4 and C6 which express altered Fur (named for ferric uptake regulation) proteins and constitutively produce the siderophores pyochelin and pyoverdin. Both mutants required a significant lag phase prior to log-phase aerobic growth, but this lag was not as apparent when the organisms were grown under microaerobic conditions. The addition of iron salts to mutant A4 and, to a greater extent, C6 cultures allowed for an increased growth rate under both conditions relative to that of bacteria without added iron. Increased manganese superoxide dismutase (Mn-SOD) and decreased catalase activities were also apparent in the mutants, although the second catalase, KatB, was detected in cell extracts of each *fur* mutant. Iron deprivation by the addition of the iron chelator 2,2′-dipyridyl to wild-type bacteria produced an increase in Mn-SOD activity and a decrease in total catalase activity, similar to the *fur* mutant phenotype. Purified wild-type Fur bound more avidly than mutant Fur to a PCR product containing two palindromic 19-bp "iron box" regions controlling expression of an operon containing the *sodA* gene that encodes Mn-SOD. All mutants were defective in both ferripyochelin- and ferripyoverdin-mediated iron uptake. Two mutants of strain PAO1, defective in pyoverdin but not pyochelin biosynthesis, produced increased Mn-SOD activity. Sensitivity to both the redox-cycling agent paraquat and hydrogen peroxide was greater in each mutant than in the wild-type strain. In summary, the results indicate that mutations in the *P. aeruginosa fur* locus affect aerobic growth and SOD and catalase activities in *P. aeruginosa*. We postulate that reduced siderophore-mediated iron uptake, especially that by pyoverdin, may be one possible mechanism contributing to such effects.**

Pseudomonas aeruginosa is an obligate respirer whose ecological niches are extremely diverse, ranging from soils and streams to those which involve pathogenesis of plants, animals, and humans. The respiratory metabolism of molecular oxygen (O_2) by this organism can result in the production of potentially toxic oxygen reduction products, including superoxide (O_2^-) and hydrogen peroxide (H_2O_2). The production of these and other oxidants by bacteria and eukaryotic cells has been linked to mutations, membrane damage, and cell death (for reviews, see references 19 and 27). Primarily through the work of Fridovich and coworkers (22, 23, 34, 35), O_2^-, a molecule thought previously to be relatively benign (20), was found to inactivate several enzymes important in branched-chain amino acid biosynthesis, oxidative defense, and the Krebs cycle. Because of the ramifications of potentially hazardous elevated levels of O_2^-, *P. aeruginosa* possesses an iron (Fe)- and a manganese (Mn)-cofactored superoxide dismutase (SOD) (26, 30, 31) encoded by the *sodA* and *sodB* loci, respectively (30, 31). One of the products of SOD is H_2O_2, which is much more hazardous to the organism than O_2^-. It is important to eliminate H_2O_2 because of its potential reactivity with ferrous iron via a Fenton reaction to form the hydroxyl radical (HO·), which possesses nearly diffusion-limited reactivity with all biomolecules. Thus, to help dispose of H_2O_2, *P. aeruginosa* possesses two catalases, one of which, KatA (~170 kDa), is produced throughout the entire growth phase (9, 26), while the other, KatB (~228 kDa), is produced when the organism is exposed to paraquat (a redox-cycling compound) or H_2O_2 (9). Since the production of HO· is dependent upon the presence of iron, it would behoove the organism to store iron in compounds whose function is to detoxify these compounds, such as iron (Fe)-SOD and catalase.

The control of iron acquisition by bacteria including *Escherichia coli* (25), *Salmonella typhimurium* (21), *Legionella pneumophila* (32), *Vibrio cholerae* (38), *Vibrio anguillarum* (57), *Campylobacter jejuni* (65), *Yersinia pestis* (53), *Neisseria meningitidis* (56), and *Neisseria gonorrhoeae* (7) is governed, in part, by the *fur* (named for ferric uptake regulatory) gene product. The Fur protein also governs the high-affinity, siderophore-mediated, iron transport systems in many bacteria (25). Fur or Fur-like proteins have also been shown to regulate the expression of several virulence factors including a Shiga-like toxin in

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TABLE 1. *P. aeruginosa* strains used in this study

P. aeruginosa strain	Relevant genotype or characteristics	Reference or source
PAO1	Wild type	5
PAO1	Wild type	C. Cox
PAO6261	*anr*^{0}	66
A2	Fur mutant, A→G, His-86-Arg	5
A4	Fur mutant, C→T, His-86-Tyr	5
C6	*anr*^{0}, Fur mutant, GC→GG, Ala-10-Gly	5
IA613	Pyoverdin deficient for both	C. Cox
CDC-5	Pyoverdin deficient for both	C. Cox
IA-5	Pyochelin deficient	C. Cox

E. coli (10) and diphtheria toxin in *Corynebacterium diphtheriae* (49). Prince et al. (48) have recently demonstrated that the *E. coli fur* gene product controls *toxA* and *regAB* expression in *P. aeruginosa* which was subsequently followed by the cloning, sequencing, and characterization of the *fur* gene from this organism (47). *P. aeruginosa fur* mutants were isolated from strain PA103 (an exotoxin A overproducer) which allowed constitutive biosynthesis of the powerful siderophores pyoverdin and pyochelin, even in the presence of 1 mM ferric chloride, which normally restricts siderophore biosynthesis. Fur-regulated genes (at least 20 in *P. aeruginosa* [46]) harbor an iron (or Fur) box containing the palindromic consensus sequence GATAATGATAATCATTATC to which the repressor (Fur-Fe^{2+}) binds when iron is plentiful (4, 45). Recently, Ochsner et al. (45) have demonstrated that Fur recognizes an iron box in the promoter regions of *pchR* (encoding a transcriptional activator for pyochelin biosynthesis) and *pvdS* (encoding a σ factor essential for activation of *pvdA* [37]). In addition to its role in the regulation of virulence factors in many organisms, Fur also regulates the expression of the *sodA* (encoding Mn-SOD) promoter of *E. coli* (13, 44, 54, 55). Under iron-limiting conditions, *E. coli* produces elevated Mn-SOD activity, while under iron-replete conditions (such as strict anaerobiosis, where iron solubility increases), no Mn-SOD is detected (43). Hassett et al. (31) have shown elevated Mn-SOD activity in mucoid, alginate-producing *P. aeruginosa* bacteria, those commonly isolated from the airways of patients with the autosomal inherited disease cystic fibrosis.

In this report, we describe the effects of mutations within the *P. aeruginosa fur* locus on various aspects of aerobic metabolism including growth, regulation of the antioxidants SOD and catalase, and sensitivity to oxygen reduction products in the well-characterized strain PAO1. The results of this study implicate a defect in siderophore-mediated iron uptake, especially that by ferripyoverdin, as an essential factor contributing to these processes.

MATERIALS AND METHODS

Strains and media. All strains used in this study are listed in Table 1. Stock cultures were stored in 10% skim milk at −80°C.

Chemicals. Methyl viologen (paraquat), hydrogen peroxide (H_2O_2), 2,2′-dipyridyl, and chromazurol S were purchased from Sigma (St. Louis, Mo.). SOD-free catalase was purchased from Boehringer Mannheim (Indianapolis, Ind.).

Bacterial growth conditions. Bacterial cultures were grown in Erlenmeyer flasks (flask volume/medium volume ratio of either 5:1 or 10:1) at 37°C with agitation at 300 rpm in either L broth (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl per liter), low-iron Trypticase soy broth dialysate (DTSB), or DTSB supplemented with 36 μM ferric sulfate as previously described (59). Microaerobic growth was achieved by growing bacteria in plastic, sealed polycarbonate jars with CampyPak Plus oxygen tension-reducing sachets (Becton Dickinson Microbiology, Cockeysville, Md.). Chromazurol S plates (50) were used to demonstrate siderophore biosynthesis and secretion, which was manifested by red halo formation around bacterial colonies. Manganese was not incorporated into growth media, since additional mutations in loci independent of *fur* which could confuse interpretation of results might occur.

Cell extracts and protein preparation. Bacteria were harvested by centrifugation at 10,000 × *g* for 15 min at 4°C. Cell pellets were resuspended either in ice-cold 50 mM potassium phosphate (KP_i) (pH 7.0 or 7.8) with and without 0.1 mM EDTA for catalase and SOD assays or in 50 mM Tris-HCl (pH 7.4) for native gel electrophoresis. The cell suspensions were disrupted at 0°C by sonication (10- to 15-s pulses at setting 20) with a Fisher Sonic Dismembrator model 300 equipped with a microtip. Sonicated fractions were examined by light microscopy to ensure >99% cell disruption. Cell debris was removed by centrifugation (100,000 × *g* for 1 h at 4°C), and the supernatants were dialyzed exhaustively (at least 4 liters) against 50 mM KP_i with and without 0.1 mM EDTA (pH 7.0 or 7.8) or 50 mM Tris-HCl (pH 7.4) prior to use. SOD was assayed by the method of Marklund and Marklund (40). Catalase was assayed by the method of Beers and Sizer (6) with 17.6 mM H_2O_2. SOD nondenaturing gels (10%) were stained by the method of Clare et al. (12). Catalase nondenaturing gels (5%) were stained by the method of Wayne and Diaz (61). H_2O_2 concentration was measured spectrophotometrically with a molar extinction coefficient of 43.6 at 240 nm prior to each experiment. Protein was measured by the method of Bradford (8) with bovine serum albumin (fraction V; Sigma) as the standard.

DNA mobility shift assays. DNA mobility shift assays were performed as previously described (45) with an end-labelled 369-bp PCR product fragment containing the iron box region upstream of the iron-regulated *P. aeruginosa fagA-fumC-orfX-sodA* operon (28, 29) and purified wild-type and mutant (A2, A4, or C6) Fur at concentrations of 0 to 200 nM. After electrophoresis for 3 h at 200 V, gels were dried and autoradiographed. The X-ray film was scanned with a UMAX scanner, and the scans were adjusted for contrast and brightness in AdobePhotoshop 3.0 (Adobe Systems, Inc.) before being imported into Intellidraw.

Siderophore production. For production of siderophores, cultures were grown at 32°C in 0.5% Casamino Acids (Difco) which was deferrated with chelex-100 (Bio-Rad) and supplemented with 0.4 mM $MgCl_2$ (CAA) (52). Pyochelin was purified from *P. aeruginosa* PAO1 by thin-layer chromatography of ethyl acetate extracts of culture supernatants as previously described (15). Pyoverdin was purified from *P. aeruginosa* PAO1 by the method of Meyer and Abdallah (41).

Iron uptake assays. Bacteria were grown in 10 ml of CAA medium to an A_{600} of 0.3 (approximately 2×10^8 viable cells per ml). Cells were centrifuged, washed once, and resuspended in 10 ml of fresh medium. After incubation at 37°C for 10 min, pyochelin uptake reactions were initiated by the addition of 2 μg of pyochelin and 0.3 μg of $^{59}FeCl_3$ (22 μg/100 μCi; Amersham Corp.) in a 100-μl volume. Pyoverdin uptake assays were performed by the method of Cornelis et al. (14). The reactions were initiated by the addition of 5 μl of ^{59}Fe and 50 μl of a 1 mM solution of purified pyoverdin. After the addition of ^{59}Fe-labelled pyochelin or pyoverdin, 1-ml samples were removed at 1-, 2.5-, 5-, 10-, and 15-min intervals, rapidly filtered onto 0.45-μm-pore-size membrane filters, and washed with 10 mM Tris-HCl (pH 7.5). The amount of ^{59}Fe retained on the filters was determined by liquid scintillation counting. Control reactions included 2 mM KCN to inhibit energy-dependent iron uptake. Aliquots were removed at intervals and filtered as described above for siderophore-mediated iron uptake reactions.

Paraquat and H_2O_2 sensitivity assays. Bacteria were grown aerobically overnight in L broth at 37°C. Suspensions of 100 μl were distributed on L-agar plates. Sterile filter paper disks (diameter, 7 mm) were impregnated with either 10 μl of 100 μM paraquat or 30% H_2O_2. The zone of growth inhibition was then recorded after a 24-h incubation at 37°C.

Statistics. Where applicable, Student's *t* test was employed to determine statistical significance ($P < 0.001$ or 0.01).

RESULTS

Analyses of mutant Fur proteins and growth rates. Prince et al. (47) isolated spontaneous *fur* mutants of *P. aeruginosa* PA103 by using a technique which scored for increased resistance to 10 mM $MnCl_2$ (24, 51). We used this same technique to isolate *fur* mutants of strain PAO1 because this strain is better characterized on a genetic and physiological level than strain PA103. These mutants, designated A4 and C6 (Table 1) produced constitutive siderophore on the basis of their red zones on chromazurol S plates and by direct siderophore assays (data not shown). The type and location (e.g., strain A4, His-86-Tyr [His at position 86 changed to Tyr]) of the mutations in these *fur* mutants used in this study are listed in Table 1. On rare occasions (<0.01%), mutant C6 reverted back to the wild type, similar to the reversion seen in *V. anguillarum fur* mutants (36). This reversion was characterized by DNA sequence analysis after PCR and subcloning of revertant *fur* DNA (5). Interestingly, the revertant amino acid serine (Gly-

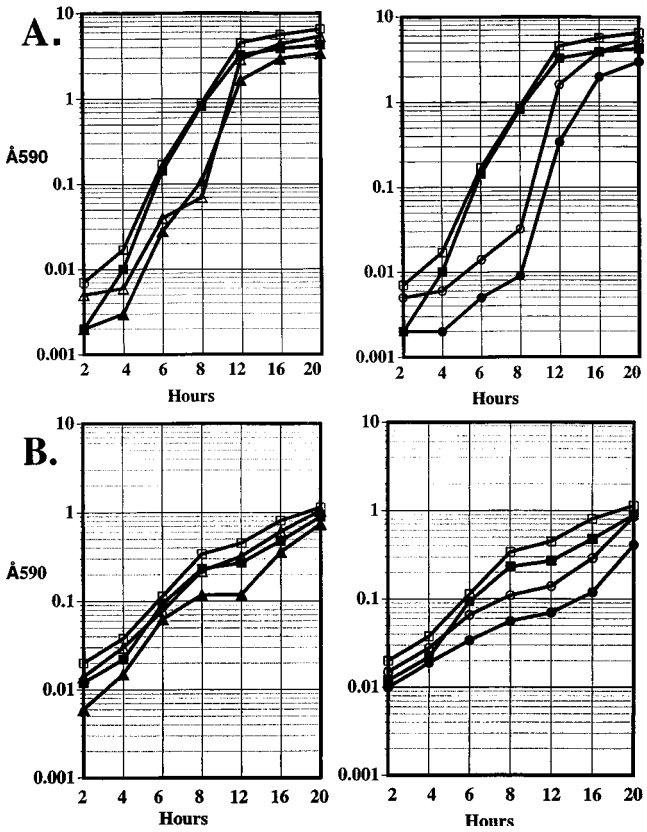

FIG. 1. Effects of altered Fur proteins on the aerobic (A) and microaerophilic (B) growth of *P. aeruginosa*. *P. aeruginosa* strains were grown overnight in DTSB medium and inoculated into fresh prewarmed DTSB with and without 36 μM ferric sulfate. Incubation was continued at 37°C, and growth was monitored until the cell density reached an A_{590} of 0.6 (~4×10^8 CFU/ml). At this point, samples were diluted 1:100 into 50 ml of fresh, prewarmed DTSB medium in 250-ml Erlenmeyer flasks, and 1.0-ml samples were withdrawn at hourly intervals and monitored for changes in A_{590}. Optical density readings greater than 1.0 were diluted 1:10 prior to absorbance readings. The results are typical of three independent experiments. *P. aeruginosa* strains were grown with and without iron as follows: PAO1 without iron (■); PAO1 with iron (□); A4 without iron (▲); A4 with iron (△); C6 without iron (●); C6 with iron (○).

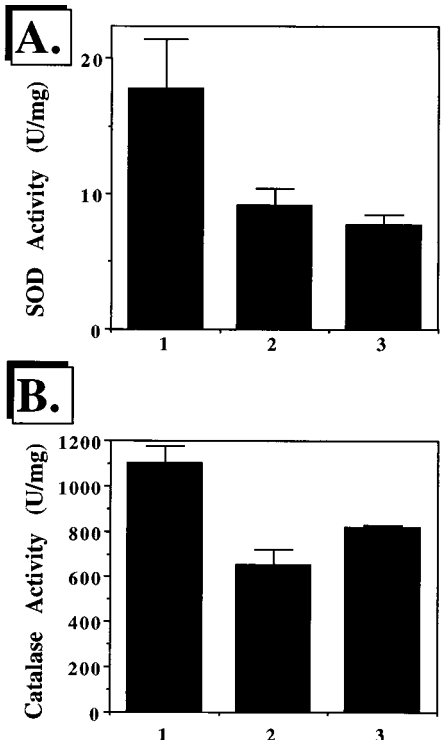

FIG. 2. SOD (A) and catalase (B) activities of *P. aeruginosa* wild-type and *fur* mutants. Bacteria were grown aerobically in L broth at 300 rpm at 37°C. Cell extracts were prepared as described in Materials and Methods. All activities are expressed as units per milligram of protein. SOD activity was measured by the method of Marklund and Marklund (40). One unit of activity was defined as a 50% inhibition of the rate of pyrogallol autoxidation. Catalase activity was measured by the method of Beers and Sizer (6) with 19 mM H_2O_2 as the substrate. The results are the means and standard errors representing three separate experiments ($P < 0.01$). Lane 1, wild-type PAO1; lane 2, A4; lane 3, C6.

10-Ser) was identical to the native *N. gonorrhoeae* Fur (7) and, thus, could substitute on a functional basis for the wild-type alanine. It should also be stressed here that the revertant behaved exactly like wild-type bacteria in all the parameters examined in this study (data not shown). In addition, strain PAO6261 (*anr*^{0}), the parent of *fur* mutant C6, was identical to strain PAO1 under all experimental conditions (data not shown).

When the *fur* mutants were grown on L-agar plates, their colonies were smaller than those of the wild type, suggesting that an altered Fur protein results in an aerobic growth defect. As shown in Fig. 1A, the growth of the *fur* mutants under aerobic conditions required a longer lag (or adaptive) period to reach the mid-logarithmic rate of wild-type bacteria. To determine whether this growth defect was dependent on oxygen, we also grew each organism under microaerobic conditions (~5% O_2). Interestingly, the aerobic growth defect was not as apparent in microaerobically grown bacteria (Fig. 1B). The addition of 36 μM ferric sulfate caused an increase in the growth rate of mutant C6 and a slight increase in mutant A4 under aerobic conditions. In contrast, these effects were approximately equal in both strains under microaerobic conditions, when iron is more soluble (62).

Mutant Fur proteins affect SOD and catalase activities and isozyme electrophoretic profile. To test the notion that the adaptive growth phenotype of the *fur* mutants may be due to reduced activities of antioxidants such as SOD and catalase, cell extracts were prepared from aerobic bacteria and examined for SOD and catalase activities. As shown in Fig. 2A, strain PAO1 produced ~18 ± 4 U of SOD activity per mg while the *fur* mutants possessed half or less activity (9.2 ± 1.2 U/mg for strain A4 and 7.7 ± 0.8 U/mg for strain C6). Catalase activity was also less in the *fur* mutants (Fig. 2B). The same cell extracts were electrophoresed and stained for SOD and catalase activities. Both of these nondenaturing gel systems were used for qualitative (e.g., demonstration of Mn-SOD and KatB expression) purposes, since the intensities of catalase (64) and SOD (this study) activity bands were not proportional to total activity. As shown in Fig. 3, the *fur* mutants (C6, lane 2 and A4, lane 3) produced higher levels of Mn-SOD than wild-type bacteria (lane 1). In addition to Fe- and Mn-SOD, *P. aeruginosa* also possesses a cytoplasmic/periplasmic ~168-kDa catalase (KatA) (26, 31) and a cytoplasmic ~228-kDa catalase (KatB) (9), both of which are inducible by H_2O_2 and paraquat (9). As shown in Fig. 4, each strain possessed KatA activity, but only the *fur* mutants demonstrated KatB activity. In this particular study, we employed another mutant A2, derived from strain PAO1 (Table 1 and Fig. 4, lane 2), which possesses a mutation (His-86-Arg) in the same amino acid as mutant A4 (His-86-Tyr). In scanning the DNA upstream of the *katB* gene (9), we found a region (CGCATTGATATTCCTAAT) at po-

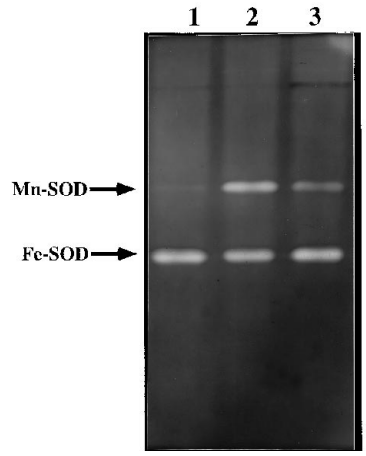

FIG. 3. SOD activity staining of aerobic cell extracts from *P. aeruginosa* wild-type and *fur* mutant strains. Bacteria were grown aerobically for 17 h in L broth at 37°C. Dialyzed cell extracts were separated by electrophoresis on a 10% native polyacrylamide gel and stained for SOD activity (12). Lane 1, wild-type PAO1; lane 2, C6; lane 3, A4.

sitions −114 to −97 relative to the ATG start codon which demonstrated a 13 of 19 match with the consensus *E. coli* iron box. However, we were unable to demonstrate a gel band mobility shift or a DNA footprint by using purified Fur and a PCR product containing this region (data not shown).

Mutant Fur proteins do not bind as avidly to the iron box region upstream of *sodA*. In two other studies, Hassett et al. (28, 29) demonstrated that the *sodA* gene, encoding Mn-SOD, is located downstream of three genes, designated *fagA* (named for Fur-associated gene), *fumC* (encoding a fumarase), and *orfX* (both *fagA* and *orfX* show no homology with any sequences in database searches). Two 19-bp palindromic iron box regions were identified immediately upstream (starting 41 bp) of *fagA*. One was located on the sense strand (5′-GAAAA CAATAATCAATCTC-3′), demonstrating a match of 14 of 19 nucleotides with the *E. coli* consensus sequence, and the other was located on the antisense strand (5′-GATAATGAGATTGA TTATT-3′), with a match of 15 of 19 nucleotides (28, 29). Because of elevated Mn-SOD activity in mutants A4 and C6, we predicted that mutant Fur proteins would bind less avidly to this region than wild-type Fur. As shown in Fig. 5 (lanes 2 to 8), increasing amounts (5 to 200 nM) of purified, wild-type Fur caused a gel mobility shift of a 369-bp PCR product containing the iron box region of this operon. The weakest *fur* mutation, on the basis of Mn-SOD expression and a retained ability to footprint the iron box region of the *pvdS* gene (5), was *fur* mutant A2. As expected, the mobility shift pattern of A2 Fur was nearly identical to that of wild-type Fur (lanes 17 to 23). In contrast, A4 Fur caused a shift with only 5 nM Fur, but the optimal shift was apparent only in the presence of >100 nM Fur. In mutant C6, the most hampered mutant (e.g., aerobic growth, increased expression of Mn-SOD and KatB, and inability to footprint the iron box region of the *pvdS* gene [5]), at least 50 nM Fur was required to cause a significant shift, and unlike wild-type, A2, and A4 Fur proteins, C6 Fur did not footprint the iron box region upstream of the *fagA-fumC-orfX-sodA*-containing operon (data not shown).

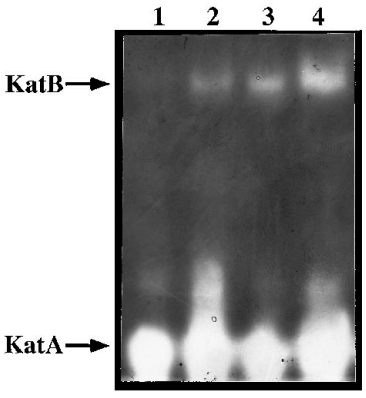

FIG. 4. Catalase activity staining of cell extracts from *P. aeruginosa* wild-type and Fur mutant strains. The cell extracts from Fig. 3 were separated on 5% nondenaturing polyacrylamide gels prior to staining for catalase activity. Samples (~5 μg) were loaded and electrophoresed at 10 mA at constant current. The gel was then stained for catalase activity (61). Lane 1, wild-type PAO1; lane 2, A2; lane 3, A4; lane 4, C6.

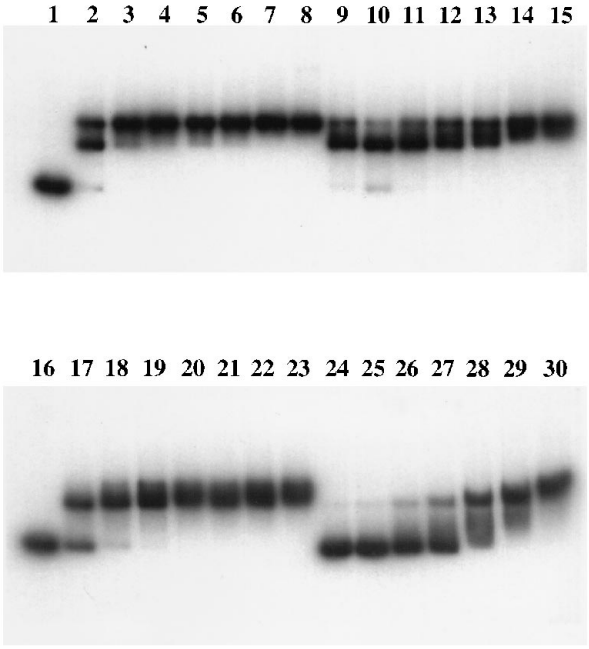

FIG. 5. Analysis of wild-type and mutant Fur proteins binding to the iron box region controlling expression of the *fagA-fumC-orfX-sodA* operon. The end-labelled 369-bp PCR product was incubated with increasing amounts of purified wild-type (lanes 2 to 8), A4 (lanes 9 to 15), A2 (lanes 17 to 23), and C6 (lanes 24 to 30) Fur. Starting from lanes 2, 9, 17, and 24, the Fur concentration increased in the order of 5, 10, 20, 30, 50, 100, and 200 nM. Lanes 1 and 16 contain no Fur.

Mutant Fur proteins affect pyoverdin- and pyochelin-mediated iron uptake. An alternative explanation for the observed effects (e.g., decreased total SOD and catalase activities and an adaptive response to aerobic growth) is a decrease in the uptake of iron by these organisms. Under conditions where iron becomes limiting, the uptake of iron by *P. aeruginosa* is facilitated by the secretion of the siderophores pyochelin (16) and pyoverdin (17). To test whether the *fur* mutants were defective in siderophore-mediated iron uptake, ferripyochelin and ferripyoverdin-mediated iron uptake studies were performed. As shown in Fig. 6, the rate of ^{59}Fe-labelled pyoverdin (Fig. 6A) and ^{59}Fe-labelled pyochelin (Fig. 6B) uptake was less in each mutant. The inclusion of 2 mM KCN in the reaction mixtures completely blocked iron uptake, confirming that the initial rate of iron accumulation is uptake and not binding of iron to the cells (data not shown).

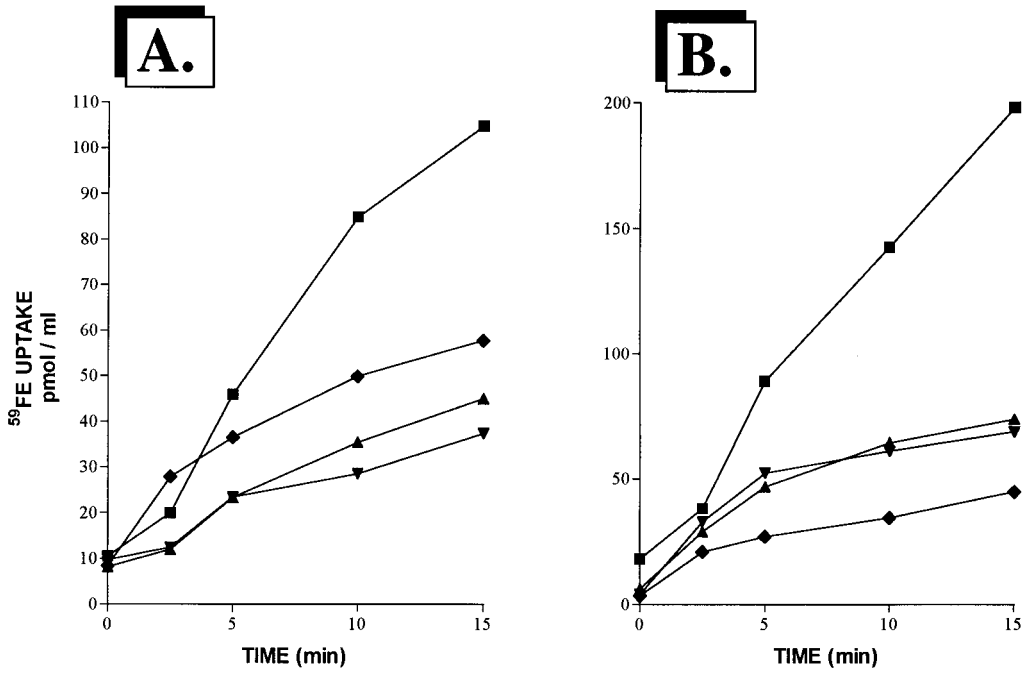

FIG. 6. Ferripyoverdin-mediated (A) and ferripyochelin-mediated (B) iron uptake by the wild type and *fur* mutants of *P. aeruginosa* PAO1. Uptake assays were initiated by the addition of ^{59}Fe-labelled pyoverdin or pyochelin. One-milliliter samples were removed at the indicated time points, and the amount of ^{59}Fe accumulated was determined. These experiments were performed at least twice with similar results. Symbols: ■, PAO1 (wild type); ▲, A2; ▼, A4; ◆, C6.

Pyoverdin- but not pyochelin-deficient mutants produce increased Mn-SOD activity. We predicted that if the level of intracellular iron plays a role in the expression of Mn-SOD, mutants defective in one or both siderophores would demonstrate increased Mn-SOD activity. As shown in Fig. 7, wild-type strain PAO1, as expected, produces only Fe-SOD (lane 1) (26, 30, 31). Similarly, the pyochelin-deficient mutant IA-5 also produced only Fe-SOD (Fig. 7, lane 4). However, two pyoverdin-deficient mutants IA613 and CDC-5 produced Mn-SOD activity (Fig. 7, lanes 2 and 3).

Iron chelation causes increased Mn-SOD and decreased catalase activities in wild-type bacteria, much like *fur* mutants. To mimic a defect in iron uptake, such as that demonstrated by the *fur* mutants in Fig. 6, we incubated wild-type *P. aeruginosa* PAO1 in the presence of increasing concentrations of the iron chelator 2,2′-dipyridyl. As shown in Fig. 8, increased Mn-SOD activity (Fig. 8A) and decreased catalase activity (Fig. 8B) was evoked by as little as 100 μM 2,2′-dipyridyl.

Mutant Fur proteins affect sensitivity to paraquat and H_2O_2. Since both total SOD and catalase activities decreased in the *fur* mutant strains of *P. aeruginosa* PAO1, we predicted that these strains would also demonstrate greater sensitivity to the redox-cycling agent paraquat and H_2O_2. We based our hypothesis on research involving *E. coli*, which is generally more resistant to redox-active compounds (e.g., paraquat) or H_2O_2 than isogenic mutants which are devoid of SOD and catalase activities (11, 39). As shown in Fig. 9, *fur* mutants A4 and C6 were more sensitive to paraquat (Fig. 9A) and H_2O_2 (Fig. 9B) than wild-type bacteria.

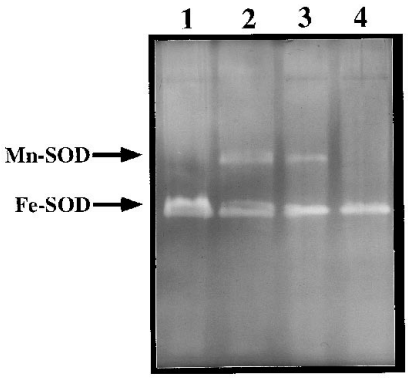

FIG. 7. Pyoverdin- but not pyochelin-deficient mutants of *P. aeruginosa* produce elevated Mn-SOD activity. Bacteria were grown for 17 h at 37°C in L broth. Dialyzed, cell extracts (40 μg) were separated by electrophoresis on 10% nondenaturing polyacrylamide gels and stained for SOD activity (12). Lane 1, PAO1; lane 2, IA613 (pyoverdin deficient); lane 3, CDC-5 (pyoverdin deficient); lane 4, IA-5 (pyochelin deficient).

DISCUSSION

Prior to this study, we had originally attempted to construct *fur* null mutants of *P. aeruginosa* to evaluate the role of Fur in the control of various cellular processes. However, despite using the most current genetic techniques, we were unsuccessful. We now know that an absence of even partially functional Fur is lethal to the organism (60). This is likely due to the fact that Fur is responsible for regulating greater than 20 genes in *P. aeruginosa* (46). In this study, we examined the effect of nonlethal missense mutations within the *fur* locus of *P. aeruginosa* PAO1 on various aspects of normal aerobic metabolism. Our initial observation was that each mutant grew more slowly than wild-type organisms and that this effect was not as pronounced in the presence of added iron or under microaerobic conditions, where iron becomes more soluble (Fig. 1) (62). These data suggested that the *fur* mutants may be defective in iron uptake and that the presence of oxygen (which decreases iron solubility) (62) contributes to this effect. An alternative explanation is that the mutant Fur proteins may have a de-

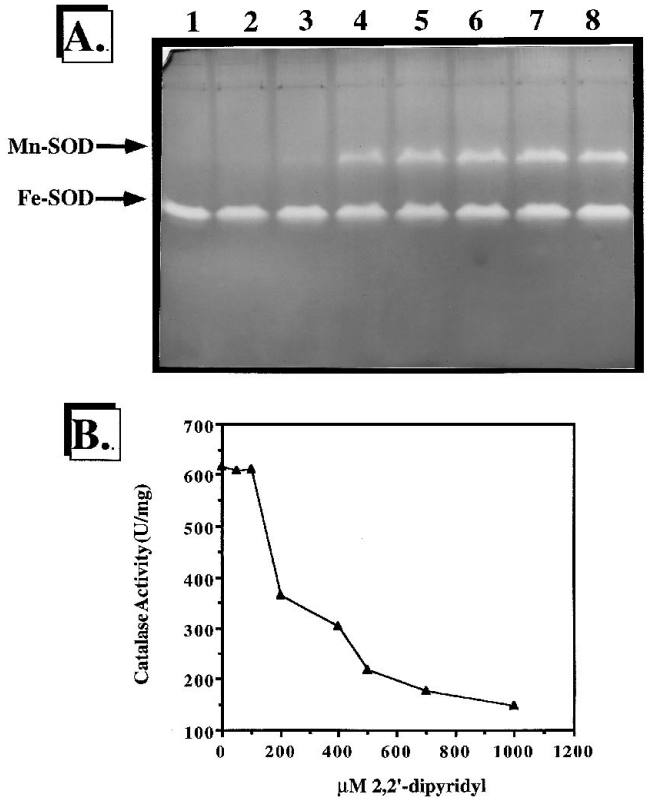

FIG. 8. Effects of 2,2′-dipyridyl on SOD and catalase activities in *P. aeruginosa* PAO1. Bacteria were grown aerobically in L broth unti the A_{600} reached 0.3. At that point, increasing concentrations of 2,2′-dipyridyl (DP) were added, the bacteria were incubated an additional 4 h at 37°C, and cell extracts were prepared as described in Materials and Methods. (A) SOD activity stain after growth in the presence of DP. Lane 1, control; lane 2, 50 μM DP; lane 3, 100 μM DP; lane 4, 200 μM DP; lane 5, 300 μM DP; lane 6, 400 μM DP, lane 7, 500 μM DP, lane 8, 600 μM DP. Approximately 70 μg of cell extract was loaded per well. (B) Catalase activity was then assayed by the method of Beers and Sizer (6). Each point is the mean of three separate experiments.

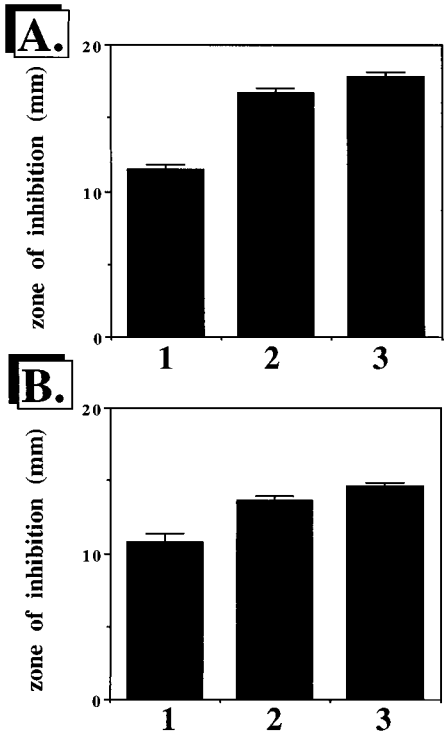

FIG. 9. Effects of *fur* mutations on the sensitivity to paraquat (A) and H_2O_2 (B) in *P. aeruginosa*. Bacteria were grown aerobically in L broth for 17 h at 37°C. Aliquots were spread on L-agar plates. Sterile 7-mm-diameter filter paper disks were impregnated with either 100 μM paraquat or 30% H_2O_2 and placed on the seeded plates. The organisms were allowed to incubate at 37°C for 24 h prior to determination of the zone of inhibition in millimeters. The results are the means and standard errors of three different experiments ($P < 0.001$ [paraquat]; $P < 0.01$ [H_2O_2]).

creased capacity to act as a positive regulator. Although known typically as a classical repressor, Fur has been observed to positively regulate a *P. aeruginosa pig* gene (named for *Pseudomonas* iron-regulated gene) (46) under iron-replete conditions (58a). A similar form of positive regulation may be operative with the *P. aeruginosa* Fe-SOD and catalase. It should also be noted that the *E. coli* Fe-SOD is positively regulated by Fur (44), the mechanism of which is unknown.

In *E. coli*, *fur* mutants produce elevated Mn-SOD activity under aerobic and anaerobic conditions (44). In this setting, an absence of Fur would allow transcription through the two iron box regions in the promoter region of the *E. coli sodA* gene (55). We recently discovered an iron box region upstream of the *P. aeruginosa fagA-fumC-orfX-sodA* operon which is upregulated under iron-limiting conditions and in alginate-producing bacteria (28, 29). Our studies demonstrate that purified mutant Fur proteins A4 and especially C6 (Fig. 5) do not bind as avidly to this region as wild-type Fur, thus allowing for increased transcription of *sodA* and concomitant Mn-SOD activity.

We also became particularly interested in the decreased catalase activity in cell extracts of the *fur* mutants. Probably because of decreased catalase activity, we also demonstrated increased sensitivity of the *fur* mutants to H_2O_2, similar to what was observed in an *E. coli fur* mutant (58). These data confirm our recent report (9) which demonstrated that elevated catalase activity is essential for optimal resistance of *P. aeruginosa* to H_2O_2. Still, despite possessing less total catalase, *fur* mutants produced elevated KatB activity relative to wild-type bacteria. KatB is a highly regulated stress protein that is not produced during normal aerobic growth in rich medium but whose activity is increased markedly upon exposure to H_2O_2 and paraquat (9). Thus, we postulated that Fur plays a role in the regulation of *katB*. However, we were unable to demonstrate a mobility shift or DNA footprint using a PCR product containing the putative iron box region (match of 13 of 19 nucleotides with those of the consensus sequence) upstream of *katB* (data not shown). Unlike the direct regulation of Fur on the *fagA-fumC-orfX-sodA* operon (28, 29; this study), Fur may be indirectly controlling *katB* expression.

Given that the *fur* mutants demonstrated (i) more rapid aerobic growth upon the addition of iron salts, (ii) increased Mn-SOD and KatB activities, and (iii) decreased total SOD and catalase activities, we postulated that these organisms are impaired in siderophore-dependent iron uptake, a factor which could prevent iron-containing proteins such as Fe-SOD and catalase from incorporating iron into their active sites. The results in Fig. 6 demonstrate that the *fur* mutants (A2, A4, and C6) are markedly impaired in both pyochelin- and pyoverdin-mediated iron uptake. To our knowledge, we know of no report demonstrating the effect of a *fur* mutation on siderophore-mediated iron uptake in other bacteria, and thus mechanisitic comparisons between organisms would be only speculative. Still, decreased siderophore-mediated iron uptake by *P. aerugi-*

nosa is in marked contrast to a *fur* mutant of *E. coli* which accumulates iron (as Fe^{2+}) at a more rapid rate than wild-type bacteria (33). In addition, Touati et al. (58) have recently shown that *fur* null mutants of *E. coli* allow an unregulated influx of iron which overwhelms the iron storage capacity of the bacterium, leading to an oxygen-dependent lethality.

It is possible that even a small difference in the rate of ferripyoverdin uptake could result in differences in the total activity and electrophoretic profile of SOD and catalase in the *fur* mutants, despite normal ferripyochelin uptake. The evidence supporting this hypothesis includes the following: (i) pyoverdin- but not pyochelin-deficient mutants produce elevated Mn-SOD activity (Fig. 7); (ii) pyoverdin has a much higher affinity constant for iron, 10^{32} (63), than pyochelin, whose constant is only $\sim 10^5$ (18); (iii) pyoverdin has been reported to be more effective at promoting the growth of *P. aeruginosa* in human serum or transferrin-containing medium (1); (iv) pyoverdin-deficient mutants grow much more slowly in the presence of 2,2′-dipyridyl than pyochelin-deficient mutants (25a); and (v) because of the higher affinity of pyoverdin for iron, more available iron would likely be complexed to pyoverdin than to pyochelin, even if both siderophores are present on an equimolar basis. Still, because the *fur* mutants produce more siderophore, it might be predicted that iron uptake by the *fur* mutants would be increased relative to wild-type bacteria. Recent evidence by Barton et al. (5) suggests that *P. aeruginosa fur* mutants secrete siderophore precursors (or immature siderophores) which may block functional siderophores from binding to their respective iron-regulated membrane receptors FptA and FpvA (2, 42). In support of this statement, Ankenbauer et al. (3) have demonstrated substantially reduced iron uptake by structural analogs of pyochelin.

In conclusion, this study demonstrates that wild-type Fur is essential for normal aerobic processes which include growth, regulation of antioxidants, and proper siderophore-mediated iron uptake. Despite an inability to generate a *P. aeruginosa fur* null mutant (60), the missense mutants employed in this study were extremely useful in gauging the role of Fur in these processes. Although still very little is known of the complex regulation of iron-regulated genes by Fur in *P. aeruginosa*, recent data by Ochsner and Vasil (46) suggest that the control of Fur-regulated genes (as a classic repressor) ranges from highly stringent to only partial control (based upon gel shift and footprint data). Thus, it is essential to pursue research avenues which focus on structure-function aspects of the *P. aeruginosa* Fur protein with target DNA. We are also currently attempting to elucidate how *fur* is regulated in *P. aeruginosa* under both oxidative and nonoxidative conditions. Such studies will contribute to our overall knowledge of oxidative processes and iron metabolism in this obligate respirer.

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