JOURNAL OF BACTERIOLOGY, Mar. 1997, p. 1452–1459 Vol. 179, No. 5
0021-9193/97/$04.00+0

An Operon Containing *fumC* and *sodA* Encoding Fumarase C and Manganese Superoxide Dismutase Is Controlled by the Ferric Uptake Regulator in *Pseudomonas aeruginosa*: *fur* Mutants Produce Elevated Alginate Levels

DANIEL J. HASSETT,[1]* MICHAEL L. HOWELL,[1] URS A. OCHSNER,[2] MICHAEL L. VASIL,[2] ZEGA JOHNSON,[2] AND GARY E. DEAN[1]

Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267-0524,[1] and Department of Microbiology, University of Colorado Health Sciences Center, Denver, Colorado 80262[2]

Received 8 November 1996/Accepted 13 December 1996

The activities of fumarase- and manganese-cofactored superoxide dismutase (SOD), encoded by the *fumC* and *sodA* genes in *Pseudomonas aeruginosa*, are elevated in mucoid, alginate-producing bacteria and in response to iron deprivation (D. J. Hassett, M. L. Howell, P. A. Sokol, M. L. Vasil, and G. E. Dean, J. Bacteriol. 179:1442–1451, 1997). In this study, a 393-bp open reading frame, *fagA* (Fur-associated gene), was identified immediately upstream of *fumC*, in an operon with *orfX* and *sodA*. Two iron boxes or Fur (ferric uptake regulatory protein) binding sites were discovered just upstream of *fagA*. Purified *P. aeruginosa* Fur caused a gel mobility shift of a PCR product containing these iron box regions. DNA footprinting analysis revealed a 37-bp region that included the Fur binding sites and was protected by Fur. Primer extension analysis and RNase protection assays revealed that the operon is composed of at least three major iron-regulated transcripts. Four mucoid *fur* mutants produced 1.7- to 2.6-fold-greater fumarase activity and 1.7- to 2.3-greater amounts of alginate than wild-type organisms. A strain devoid of the alternative sigma factor AlgT(U) produced elevated levels of one major transcript and fumarase C and manganase-cofactored SOD activity, suggesting that AlgT(U) may either play a role in regulating this transcript or function in some facet of iron metabolism. These data suggest that the *P. aeruginosa fagA*, *fumC*, *orfX*, and *sodA* genes reside together on a small operon that is regulated by Fur and is transcribed in response to iron limitation in mucoid, alginate-producing bacteria.

Pseudomonas aeruginosa produces a viscous, mucoid exopolysaccharide called alginate within the cystic fibrosis (CF) airways, an event which severely complicates the clinical scenario for CF patients (7). In recent years, multiple environmental factors have been shown to increase either the frequency of mucoid organisms from nonmucoid progenitors (59, 60) or total alginate production and *algD* gene transcription (6, 18). One of these factors, molecular oxygen, is required for optimal alginate production in vitro (4, 38). However, in inorganic solutions, elevated oxygenation also decreases the solubility of available iron (61), a metal essential for a variety of cellular processes. In an elegant chemostat study, nonmucoid *P. aeruginosa* cells grown in iron-limited media were shown to convert to the mucoid form (60). Despite this finding, the role of iron in fundamental physiological processes relating to mucoidy in *P. aeruginosa* is poorly understood.

We (32) have previously shown that mucoid, alginate-producing organisms produce elevated levels of the manganese-cofactored superoxide dismutase (Mn-SOD) (32). In the accompanying report, the *fumC* gene, encoding a tricarboxylic acid (TCA) cycle fumarase (EC 4.2.1.2), and a 444-bp open reading frame called *orfX* were localized upstream of the *sodA* gene, encoding Mn-SOD (30). Like Mn-SOD activity, fumarase activity and *fumC* mRNA levels are elevated in mucoid bacteria (30) and when organisms are exposed to iron-chelating agents (30). Interestingly, bacteria that harbor missense mutations in the *fur* gene (encoding the ferric uptake regulatory [Fur]) protein) also produce elevated Mn-SOD activity (31). Fur is a global regulator that acts as a classical DNA-binding repressor, using Fe^{2+} (and other metals [46]) as a corepressor (29). Elevated Mn-SOD in *fur* mutants suggests that in *P. aeruginosa* Fur controls transcription of *sodA*, which is part of at least one iron-regulated transcript also containing *fumC* and *orfX* (30). However, there was no consensus iron box (or Fur binding site) immediately upstream of either *sodA* (32), *orfX*, or *fumC* (30). The iron box consensus sequence GATAATGATAATCATTATC has been deduced in various Fur-regulated genes in *Escherichia coli* (9). Furthermore, Ochsner and Vasil (47) have recently identified at least 20 iron-regulated genes in *P. aeruginosa*, the promoter regions of which harbor a slightly modified form of the *E. coli* consensus sequence. In that study, it was determined that a 13-of-19-base match with the consensus sequence is necessary to demonstrate Fur control of that particular gene (using gel mobility shift and DNA footprinting analysis) (47).

Because FumC activity was absolutely essential for optimal alginate production (30), it is also possible that genes involved in alginate regulation take part, either directly or indirectly, in the control of *fumC-orfX-sodA*. The alternative sigma factor AlgT(U) (17, 19–21, 40, 41), a σ^E homolog (34), is a global regulator controlling multiple genes involved in alginate biosynthesis, including *algD*, *algZ*, *algB*, *algC*, *algR* (*algR1*), *algP* (*algR3*), and *algQ* (*algR2*) (5, 27, 62, 63). To date, there is no evidence of AlgT(U) control of genes that are not involved in alginate-related functions.

* Corresponding author. Mailing address: Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine, 231 Bethesda Ave., Cincinnati, OH 45267-0524. Phone: (513) 558-1154. E-mail: hassetdj@popmail.uc.edu.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or characteristics[a]	Source or reference
E. coli		
HB101	*proA2 leuB6 thi-1 lacY1 hsdR hsdM recA13 supE44 rpsL20*	H. Boyer
DH5α	F^- *lacZ*ΔM15 *recA1 hsdR17 supE44* Δ(*lacZYA argF*)	Bethesda Research Laboratories
SM10	Mobilizer strain	57
P. aeruginosa		
FRD1	Prototrophic, Fur^+, mucoid CF isolate	26
FRD440	*algT*::Tn*501-33*	19
FRD1 (*fur1*)	Mn^r, *fur* mutant	This study
FRD1 (*fur7*)	Mn^r, *fur* mutant	This study
FRD1 (*fur8*)	Mn^r, *fur* mutant	This study
FRD1 (*fur9*)	Mn^r, *fur* mutant	This study
Plasmids		
pUC18	Ap^r	This laboratory
pBluescriptKS−/+	Extended polylinker pUC derivative, Ap^r	Stratagene
pCRII	PCR cloning vector	InVitrogen
pNOT19	pUC19 + *Not*I site, Ap^r	53
pMOB3	*sacB* Cm^r Km^r *oriT mob*	53
pUCGM	pUC19 + 850-bp Gm^r cassette	54
pDJH120	Ap^r, *Not*I-*Pst*I fragment containing the *P. aeruginosa fagA* gene in pNOT19	This study
pDJH121	pDJH120 linearized with *Not*I and ligated to the 5.8-kb Cm^r *oriT sacB* region of pMOB3	This study

[a] Abbreviations used for genetic markers were as described by Holloway et al. (35). *mob*, mobilization site (ColE1); *oriT*, origin of transfer (RK2); Mn^r, manganese resistance; Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Gm^r, gentamicin resistance; Km^r, kanamycin resistance.

In this study, we have cloned a gene, *fagA* (Fur-associated gene), which is the first gene of a ~2.9-kb iron-regulated operon that contains *fumC*, *orfX*, and *sodA*. This operon was found to be under direct control of Fur and indirectly by AlgT(U), suggesting that cellular redox status, mediated by iron homeostasis, may be an important factor for optimal alginate production by this organism.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. All *P. aeruginosa* and *E. coli* strains used in this study are listed in Table 1 and were maintained on Luria (L) agar plates containing 10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, and 15 g of Bacto Agar per liter. All strains were stored indefinitely at −80°C in 10% skim milk.

Chemicals. 2,2′-Dipyridyl, bovine serum albumin (BSA; fraction V), poly(dI-dC), $MnCl_2$, ampicillin, and chloramphenicol were purchased from Sigma Chemical Co. Hydrogen peroxide and gentamicin were from Fisher Scientific. All other chemicals were of reagent grade or better.

Growth conditions. All bacteria were grown from single-colony isolates or overnight cultures in L broth (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter). Liquid cultures were grown at 37°C with shaking at 300 rpm or on a roller wheel at 70 rpm unless otherwise indicated, and media were solidified with 1.5% Bacto Agar. Culture volumes were 1/10 of the total Erlenmeyer flask volume to ensure proper aeration.

Cloning and sequence analysis of *fagA*. Steps involved in the cloning of the *P. aeruginosa* FRD *fagA* gene are described in Results. All DNA sequencing was performed by using the dideoxy method on double-stranded DNA (Sequenase 2.0; U.S. Biochemical, Cleveland, Ohio). Sequence obtained by this method was confirmed on both strands by using a PRISM Dye Deoxy Terminator Cycle Sequencing kit and analyzed on an ABI model 373A DNA Sequencer. Oligonucleotides for DNA sequencing reactions and PCR analysis were synthesized in the DNA Core Facility in the Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine.

Manipulation of recombinant DNA. DNA transformations were performed with *E. coli* DH5α-MCR (Gibco-BRL, Gaithersburg, Md.) and SM10 (57) as plasmid recipients. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 40 μg/ml) was routinely added to agar medium to detect the presence of insert DNA. Restriction endonuclease, alkaline phosphatase, and T4 DNA ligase were used as specified by the vendor (Gibco-BRL). Plasmid DNA was isolated by the alkaline lysis method described by Maniatis et al. (39). Restriction fragments were recovered from agarose gels by using SeaPlaque low-melting-point agarose (FMC BioProducts, Rockland, Maine) or a GeneClean II kit (Bio 101, Inc., La Jolla, Calif.).

DNA mobility shift assays. The method described by de Lorenzo et al. (14) was used, with minor modifications. A ^{32}P-labeled 369-bp *Sau*3A fragment containing 263 bp upstream and 95 bp downstream of the Fur box (iron box [Fig. 1C]) upstream of the *fagA* gene was mixed with 20 μl of binding buffer composed of 10 mM bis-Tris borate (pH 7.5), 40 mM KCl, 0.1 mM $MnSO_4$, 1 mM $MgSO_4$, 0.1 mg of BSA (fraction V; Sigma) per ml, 50 μg of poly(dI-dC) (Sigma) per ml, and 10% glycerol. Purified Fur protein was added to a final concentration of 0 to 300 nM, and the mixtures were incubated for 15 min at 37°C. A 5% nondenaturing polyacrylamide gel prepared in running buffer (20 mM bis-Tris borate, 0.1 mM $MnSO_4$) was prerun for 10 min at 200 V and loaded with 10 μl of the binding reaction without dye. After electrophoresis for 3 h at 200 V, the gel was dried and autoradiographed.

DNase I footprint analysis. The protocol for DNase I footprint experiments using *E. coli* Fur (14) was modified as follows. Increasing amounts of *P. aeruginosa* Fur protein (0 to 300 nM) were mixed with 50 μl of footprint binding buffer composed of 10 mM bis-Tris (pH 7.0), 0.1 mM $MnSO_4$, 50 mM KCl, 1 mM $CaCl_2$, 0.25 mM dithiothreitol, 0.1 mg of BSA per ml, 10 μg of poly(dI-dC) per ml, and 5% glycerol. After 10 min at room temperature, 1 μl of the end-labeled DNA fragment (2 to 10 ng) was added, and the mixtures were incubated for an additional 15 min at room temperature. DNase I treatment was performed by adding 50 μl of DNase I solution containing 50 ng of DNase I (Sigma) per ml in 10 mM HEPES (pH 7.8)–5 mM $MgCl_2$–25 mM NaCl–1 mM $CaCl_2$. The reactions were stopped after 1 min by placing the tubes in a dry ice-ethanol bath. The DNA was precipitated with 3 volumes of ethanol containing 1 M ammonium acetate and 1 μg of tRNA per ml for 20 min in dry ice-ethanol, recovered by centrifugation, washed with 80% ethanol, dried, and redissolved in 5 μl of formamide gel loading buffer. Samples were heated for 5 min at 90°C prior to loading on a 5% denaturing polyacrylamide gel.

Primer extension analysis. For primer extension analysis of the *fagA-fumC-orfX-sodA* operon, total cellular RNA was isolated from logarithmic-phase cells (optical density at 600 nm of 0.3) of *P. aeruginosa* FRD1 and FRD2 grown in L broth with or without 0.5 mM 2,2′-dipyridyl as described previously (30). Synthetic oligonucleotides specific for the iron box region upstream of *fagA* (transcript 1 [T1]) (5′-CCAGTACCAGTGATCCAATGCTT-3′), a transcript starting within *fagA* (T2) (5′-GAAGCTCATCGACGGCGCCA-3′), and *fumC* (T3 and T4) (5′-CCATGCTGTCGCGTTCGATG-3′) were end labeled with [γ-^{32}P]ATP by polynucleotide kinase as described previously (3) and incubated with 25 μg of RNA for 5 min at 85°C and 3 h at 42°C to permit annealing. This was followed by extension with 200 U of reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, Md.) in the presence of deoxynucleoside triphosphates (0.2 mM) for 30 min at 42°C. The extended DNA product was isolated and then analyzed by electrophoresis on 8% denaturing polyacrylamide gels as described previously (3). Dideoxynucleotide sequencing reactions, primed from the same end-labeled oligonucleotide, were electrophoresed in adjacent lanes to identify the start of transcription.

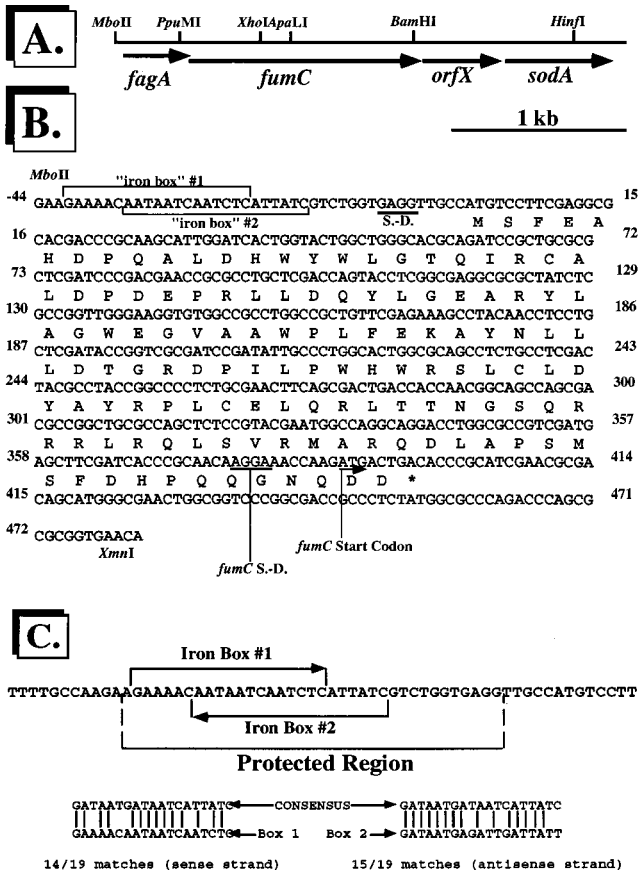

FIG. 1. Restriction map of the *fagA-fumC-orfX-sodA* operon. (A) Appropriate restriction sites are shown. (B) DNA sequence of *fagA* of *P. aeruginosa* FRD. The ribosome binding (Shine-Dalgarno [S.-D.]) sequence is underlined. The *fagA* stop codon is marked by an asterisk. The sense-strand iron box is box 1, and the complementary strand harbors box 2. (C) Location of the *P. aeruginosa* Fur iron boxes upstream of *fagA* and homologies with the *E. coli* consensus iron box.

Preparation of RNA probes and RNase protection assays. A DNA fragment covering potential transcriptional initiation sites was generated by PCR using *P. aeruginosa* FRD2 genomic DNA as the template and the following primers: forward primer, 5′-TTGTCCGCCTCTATGGGGA-3′; and reverse primer, 5′-CAGCAGTTGCAGGCAGGTGT-3′. This 713-bp PCR product was cloned into pCRII (InVitrogen), which carries the SP6 and T7 promoters. The plasmid was linearized either with *Not*I (when the SP6 promoter was used) or with *Hin*dIII (when the T7 promoter was used). RNA complementary to each DNA strand was produced by using a Riboprobe kit (Promega Corp., Madison, Wis.) according to the manufacturer's specifications. Probes were hybridized with 20 μg of total cellular RNA. RNase protection assays were performed according to the procedure described by Sambrook et al. (50), which is a modification of the method of Melton et al. (42). RNA-RNA hybrids were heated for 5 min at 95°C in formamide loading buffer (80% formamide, 10 mM Na_2EDTA [pH 8.0], 1 mg of xylene cyanol per ml, 1 mg of bromophenol blue per ml) prior to electrophoresis on a 5% polyacrylamide–7 M urea sequencing gel. Following electrophoresis, the gel was dried on Whatman 3MM paper (Whatman Laboratory Products, Clifton, N.J.) and exposed to X-ray film without an intensifying screen. Radiolabeled RNA molecular weight standards were prepared by labeling of the 5′ ends of a 0.16- to 1.77-kb RNA ladder (Gibco-BRL) with T4 polynucleotide kinase (Gibco-BRL) and [γ-^{32}P]ATP after dephosphorylation of the 5′ ends with calf intestine alkaline phosphatase (Gibco-BRL).

Isolation of *P. aeruginosa* FRD1 *fur* mutants. In previous studies, we used a manganese mutagenesis technique (56) to isolate *fur* mutants of *P. aeruginosa* PAO1 (31) and PA103 (48). The wild-type phenotype could easily be restored by expressing the *fur* gene in *trans* (data not shown) or by rare reversion events. Mucoid *P. aeruginosa* FRD1 was grown aerobically for 17 h in L broth. Suspensions (100 μl) were distributed on agar plates containing 4 g of tryptone, 2.5 g of glucose, and 10 mM $MnCl_2$ per liter (56) and incubated for 48 to 72 h at 37°C. Manganese-resistant bacteria were streaked for isolated colonies on chromazurol S plates (55), which have been used previously to demonstrate constitutive siderophore biosynthesis, a process controlled by the *fur* gene product (31, 48, 49). Colonies producing a red halo were found to possess mutated *fur* genes by PCR and sequencing of the *fur* genes. Four *fur* mutants (Table 1) were used in this study.

Purification of Fur, cell extract preparation, and biochemical assays. The *P. aeruginosa* Fur protein was purified as previously described (46). Cell extracts of mid-logarithmic-phase organisms or overnight-grown bacteria were prepared from cultures harvested by centrifugation at 10,000 × *g* for 10 min at 4°C. Bacteria were washed twice in ice-cold 50 mM sodium phosphate buffer (pH 7.0) and sonicated in an ice water bath for 10 s with a Fisher Scientific model 50 Sonic Dismembrator equipped with a microtip at output setting 20. The sonicate was then clarified by centrifugation at 13,000 × *g* for 10 min at 4°C. Fumarase (EC 4.2.1.2) was assayed spectrophotometrically at 250 nm while the conversion of L-malate to fumarate at 25°C was recorded (33). Cell extract preparation for native gel electrophoresis was performed as described above except that 50 mM Tris-HCl (pH 7.4) was used as the diluent. Native gels (10% polyacrylamide) were stained for SOD activity as described by Clare et al. (12). Alginate in water-dialyzed *P. aeruginosa* culture filtrates was assayed as described by Knutson and Jeanes (37). Protein concentrations in cell extracts were estimated by the method of Bradford (8), using BSA as the standard.

GenBank accession number. GenBank accession number U59458 has been assigned to the *fagA* gene as well as the *fumC* gene (30). The remainder of the operon has been assigned accession number L25672A (32).

RESULTS

Sequence analysis upstream of the *P. aeruginosa fumC* gene: identification of *fagA*. In our accompanying report (30), we demonstrated that the *fumC* gene, encoding a TCA cycle fumarase, was located upstream of the *orfX* and *sodA* genes in *P. aeruginosa*. Because (i) fumarase activity and *fumC* transcription were increased in response to iron deprivation (30) and (ii) Mn-SOD activity is elevated in *fur* mutants (31), we postulated that the *fur* gene product may play a role in the regulation of both *fumC* and *sodA* in this organism. However, there was no consensus iron box region (or Fur binding site) upstream of either *fumC* (30), *orfX*, or *sodA* (32). When we sequenced an additional ~1.5 kb upstream of the *fumC* start codon (a restriction map of the complete iron-regulated operon is shown in Fig. 1A), we discovered a 393-bp open reading frame (*fagA* [Fig. 1B]) that started at nucleotide 1 and terminated at nucleotide 393. The 3′ end of *fagA* was found to overlap the *fumC* start codon by 6 bp. A GenBank search for nucleotide or translated *fagA* yielded no homology matches with genes or proteins in several databases.

Demonstration of an iron box upstream of *fagA*. A consensus iron box sequence of GATAATGATAATCATTATC has been determined in *E. coli* based on promoter region sequences to which Fur binds under iron-replete conditions (9). When we inspected the sequence immediately upstream of the *fagA* gene, we found two putative iron box regions encompassing nucleotides −41 to −17 (Fig. 1C). Iron box 1 is in the sense orientation and possesses a 14-of-19-base match with the *E. coli* consensus iron box region; iron box 2 is in the antisense orientation and possesses a 15-of-19-base match with the consensus sequence (Fig. 1C).

Gel mobility shift and DNase I footprinting analysis of the *fagA* iron box region. To determine if *P. aeruginosa* Fur binds to the two putative iron boxes depicted in Fig. 1C, we first performed gel band mobility shift assays after incubating increasing concentrations (1 to 300 nM) of purified *P. aeruginosa* Fur with a ^{32}P-labeled 369-bp PCR product harboring the putative iron box regions upstream of *fagA*. Purified *P. aeruginosa* Fur caused a gel band mobility shift of the PCR fragment in assays using as little as 10 nM of purified Fur protein (Fig. 2, lane 4). The addition of polyclonal anti-Fur antibody to reaction mixtures caused a supershift of the Fur-DNA complex (Fig. 2, lane 8). To determine precisely where Fur binds within this region, DNase I footprinting analysis was performed with the same PCR product as used for the gel mobility shift assays. Consistent with the mobility shift assays, 10 nM Fur protected

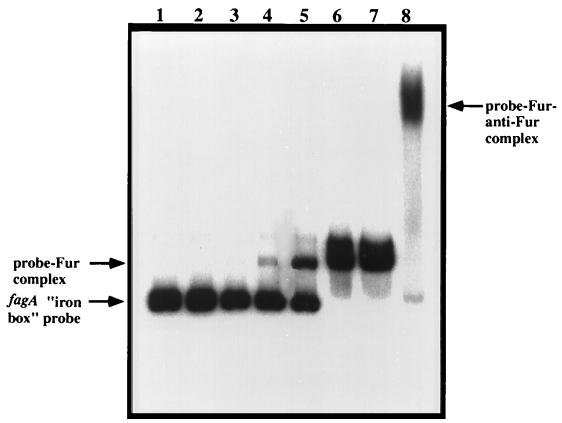

FIG. 2. Analysis of Fur binding to potential Fur binding sites upstream of the *fagA* gene of *P. aeruginosa*, using a gel band mobility shift assay. An end-labeled 369-bp *Sau*3A fragment containing 263 bp upstream and 95 bp downstream of the Fur box (iron box 2 [Fig. 1C]) was incubated with various quantities of purified *P. aeruginosa* Fur protein. Lane 1, no Fur; lane 2, 1 nM Fur; lane 3, 3 nM Fur; lane 4, 10 nM Fur; lane 5, 30 nM Fur; lane 6, 100 nM Fur; lane 7, 300 nM Fur; lane 8, 100 nM Fur plus polyclonal antiserum raised against purified *P. aeruginosa* Fur.

a 37-bp region encompassing nucleotides −42 to −6 (AGAA AACAATAATCAATCTCATTATCGTCTGGTGAGG) (Fig. 3 and 1C).

Primer extension analysis of *fagA-fumC-orfX-sodA*. In our accompanying report (30), we demonstrated that *fumC*, *orfX*, and *sodA* are part of at least one iron-regulated transcript. To further characterize the regulation of this region, the start sites of transcription were determined by primer extension analysis. Because mucoid bacteria demonstrated elevated transcription of *fumC* (30) relative to nonmucoid strain FRD2, which harbors an *algT(U)* missense mutation (*algT18* [19]), we postulated that the alternative sigma factor AlgT(U) (19, 40) might also play a role in the regulation of this operon. RNA was prepared from mucoid strain FRD1, strain FRD2, and strain FRD440 (*algT*::Tn*501-33* [63]). Strain FRD440 is completely devoid of any functional AlgT(U) (47a). As shown in Fig. 4A, the primer extension analysis revealed at least four iron-regulated transcripts (T1 to T4) within or upstream of the *fagA-fumC-orfX-sodA* operon. The first transcript (T1) starts at a thymine residue (Fig. 4B, left panel) within the iron box region upstream of *fagA* and includes the entire operon. Interestingly, the amount of transcript T1 is greatest in strain FRD440 (*algT*::Tn*501-33*). Transcript T2 (site within *fagA* [Fig. 4B, middle panel]) starts at an adenine residue 22 bp downstream of what we postulated was a consensus −35 promoter region (GAACTT) for AlgT(U)-regulated genes (27). This consensus sequence was previously found in the −35 regions of the *algT(U)*, *algR*, *algD*, and *rpoH* of *P. aeruginosa* by Schurr et al. (51). However, AlgT(U) does not appear to control transcript T2 since a primer extension product is present in RNA extracted from strain FRD440 (*algT*::Tn*501-33*). Transcripts T3 and T4 start at an adenine and a guanine at positions −11 and −9, respectively, relative to the start codon of *fumC* (Fig. 4B, right panel). Interestingly, both T3 and T4 are within the putative ribosome binding site of *fumC*, with transcript T3 being the most prominent of the two. These results were confirmed by RNase protection assays (data not shown).

An *algT(U)* mutant produces elevated Mn-SOD activity: evidence that AlgT(U) may be involved in iron metabolism. Since the *algT(U)* knockout mutant (strain FRD440) demonstrated elevated transcript T1, we postulated that an additional role(s)

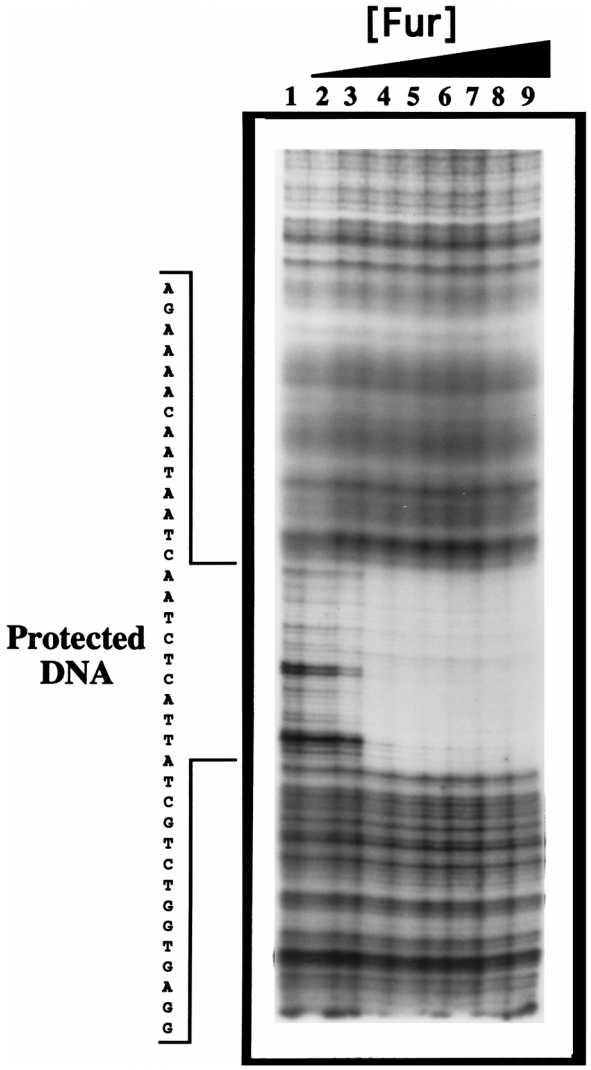

FIG. 3. DNase I footprinting of purified *P. aeruginosa* Fur protein on the Fur binding sites upstream of the *fagA* gene of *P. aeruginosa*. Lane 1, no Fur; lane 2, 2 nM Fur; lane 3, 5 nM Fur; lane 4, 10 nM Fur; lane 5, 20 nM Fur; lane 6, 40 nM Fur; lane 7, 80 nM Fur; lane 8, 150 nM Fur; lane 9, 300 nM Fur.

of AlgT(U) might be as a negative regulator of T1 and/or in the control of unknown gene(s) involved in iron metabolism. This is not unprecedented, since Yu et al. (65) have shown by two-dimensional gel electrophoresis that multiple proteins, the identities of which are currently unknown, are both up- and downregulated by AlgT(U). As shown in Fig. 5, mucoid *P. aeruginosa* produced more Mn-SOD (lane 1) than did nonmucoid bacteria (lane 2). Interestingly, the *algT(U)* knockout mutant produced Mn-SOD levels comparable to or greater than those of wild-type mucoid bacteria (lane 3).

***fur* mutants of mucoid *P. aeruginosa* possess elevated fumarase activity and alginate levels.** Since we demonstrated that *P. aeruginosa* Fur binds to the iron boxes upstream of *fagA*, we predicted that organisms harboring a mutation in the *fur* locus would possess elevated fumarase activity. To test this hypothesis, we isolated four *fur* mutants of *P. aeruginosa* FRD1 after manganese mutagenesis (48, 56). This technique has been successfully used to generate *fur* mutants of *P. aeruginosa* PA103 (48) and, more recently, the well-characterized strain PAO1

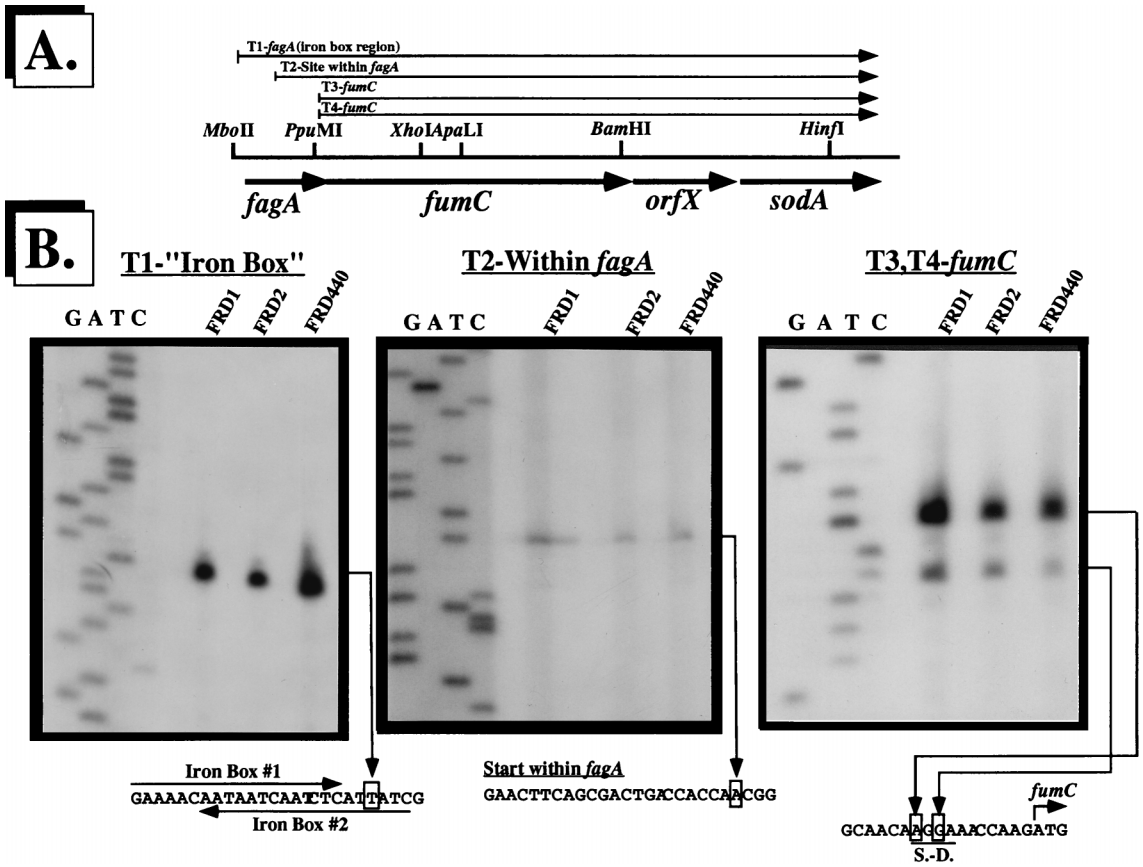

FIG. 4. Primer extension analysis of the *fagA-fumC-orfX-sodA* operon. (A) Restriction map of the *fagA-fumC-orfX-sodA* operon showing the locations of transcriptional start sites (T1 to T4). An arrow indicates the size of each transcript that was confirmed by Northern blot analysis (data not shown). (B) RNA was purified from mid-log-phase *P. aeruginosa* FRD1, FRD2 [*algT(U)18*], and FRD440 [*algT(U)*::Tn*501-33*] that had been treated with 0.5 mM 2,2′-dipyridyl for 3 h. Oligonucleotides were end labeled with ^{32}P and used in a primer extension reaction with 25 μg of purified RNA as described in Materials and Methods.

(31). A mutation in the *fur* locus was confirmed after growth of potential candidates on chromazurol S plates, which revealed a characteristic red halo surrounding positive *fur* mutants (48). This red halo is a hallmark of constitutive siderophore (pyochelin and pyoverdin) biosynthesis by this organism. As shown in Fig. 6A, each of the *fur* mutants (lanes 2 to 5) possessed ~1.7- to 2.6-fold-greater fumarase activity than *P. aeruginosa* FRD1 (lane 1).

In our accompanying report (30), we demonstrate that *fumC* mutants of mucoid *P. aeruginosa* produce only ~40% of the alginate of wild-type bacteria. Thus, the *fumC* gene product is essential for optimal alginate production by *P. aeruginosa*. We postulated that *fur* mutants, being derepressed for FumC activity, would produce greater alginate levels than wild-type bacteria. As shown in Fig. 6B, each of the *fur* mutants used for Fig. 6A produced 1.7- to 2.3-fold-greater alginate levels than wild-type bacteria.

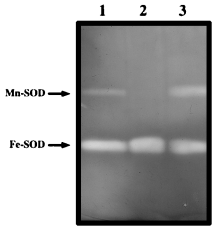

FIG. 5. Native polyacrylamide gel electrophoresis of *P. aeruginosa* cell extracts stained for SOD activity. Duplicate samples of *P. aeruginosa* FRD1 (lane 1), FRD2 (*algT18*) (lane 2), and FRD440 (*algT*::Tn*501*-33) (lane 3) were grown aerobically in L broth for 17 h. Cell extracts were prepared as described in Materials and Methods, and 50 μg of protein was subjected to native gel electrophoresis and SOD activity staining (12).

DISCUSSION

Alginate production by *P. aeruginosa* involves a complex series of events governed by >25 gene products. These genes (called either *alg* [alginate] or *muc* [mucoidy]) are involved in the regulation (15, 19, 36, 40, 45, 52, 63, 64), biosynthesis (16, 66), structure (10), acetylation (22, 23), and secretion (11) of alginate. A major research focus has been to determine the environmental factors that trigger conversion to mucoidy (6, 18, 59, 60) or maintenance of mucoidy in vivo; two in vitro factors are oxygen (4, 38) and iron deprivation (60). Oxygen is absolutely required for optimal alginate production (4, 38), while iron deprivation has been shown to increase the frequency of mucoid subpopulations from nonmucoid progenitors in chemostat culture (59, 60).

Hassett et al. (32) have previously demonstrated that mucoid bacteria produce elevated Mn-SOD and total SOD activity relative to nonmucoid bacteria and, as shown in our accompanying report (30), elevated FumC activity. These events are triggered by iron limitation, since (i) FumC and Mn-SOD

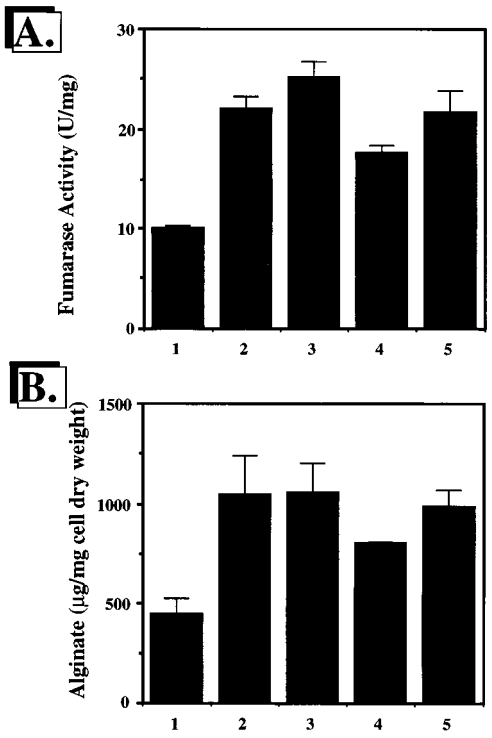

FIG. 6. Fumarase activity (A) and alginate production (B) of wild-type and *fur* mutant *P. aeruginosa* FRD1. *fur* mutants of *P. aeruginosa* FRD1 were isolated as described in Materials and Methods (48, 56). All bacteria were grown aerobically in L broth at 37°C for 17 h prior to preparation of cell extracts and fumarase activity measurements (33). Culture supernatants were dialyzed exhaustively against distilled water, and uronic acid alginate was quantified as described by Knutson and Jeanes (37). Lane 1, FRD1; lane 2, FRD1 (*fur1*); lane 3, FRD1 (*fur7*); lane 4, FRD1 (*fur8*); lane 5, FRD1 (*fur9*).

activity and *fagA-fumC-orfX-sodA* transcript levels are upregulated upon exposure to the hydrophobic iron chelator 2,2′-dipyridyl (Fig. 4) (30) and (ii) *fur* mutants of strain PAO1 also produce elevated Mn-SOD activity (31). One clue that would explain why siderophore (pyochelin and pyoverdin) levels and FumC and Mn-SOD activities are higher in mucoid than in nonmucoid bacteria (30) was found in the identification of two iron box regions in the promoter region upstream of the *fagA* gene. Definitive evidence of Fur control of this region was demonstrated by gel mobility shift and DNA footprinting experiments (Fig. 2 and 3). These techniques were recently used by Ochsner and Vasil (47) to identify at least 24 Fur-regulated genes in *P. aeruginosa* PAO1. Interestingly, there are two iron boxes in the promoter region of the *sodA* gene of *E. coli*, which is also under Fur control (13, 58).

The regulation of the *fagA-fumC-orfX-sodA* operon is complex. Both primer extension analysis (Fig. 4) and RNase protection assays (data not shown) confirmed that there are at least four transcriptional start sites, one that begins upstream of the *fagA* gene, one that begins within *fagA*, and two others that begin upstream of *fumC*. Upon scanning the DNA sequence upstream of *fumC*, we found a consensus −35 promoter sequence (GAACTT) for the AlgT(U) protein (but no −10 consensus), a σ^E homolog that is absolutely required for alginate biosynthesis (19, 21, 27, 40), within the *fagA* coding sequence. This is not uncommon since AlgT(U) promoters have also been found in the coding region of *algZ*, a gene immediately upstream of *algR* (14a, 27). Because an *algT(U)* insertional mutant still produced this transcript, it was clear that AlgT(U) did not play a role in the regulation of this particular transcript. Thus far, AlgT(U) is known only to control alginate-related genes (27). However, transcript T1 levels (Fig. 4) and Mn-SOD activity (Fig. 5) were markedly elevated in strain FRD440 [*algT(U)*::Tn*501-33*], which suggested that AlgT(U) may also participate in the regulation of the *fagA-fumC-orfX-sodA* operon and/or genes involved in iron metabolism or acquisition. This hypothesis is currently under investigation.

Currently, the role of iron in alginate production is poorly understood. Since the addition of iron chelators to mucoid or nonmucoid bacteria did not increase *algD* transcription in vitro (29a), it is possible that iron deprivation is not a major factor in mucoid conversion in vivo. Although iron deprivation may contribute to this process in vivo, other events, such as mutations in alginate-negative regulatory loci (i.e., *mucA* [41] or *mucB* [40, 41], which is also called *algN* [25]) or unappreciated CF airway factors, may collectively lead to mucoid conversion.

It is believed that alginate production is an energy-expensive process (44). Still, virtually nothing is known of the bioenergetics (cytochrome levels and activities, electron transport/oxidative phosphorylation, and proton pumping) of alginate production. One possibility is that mucoid organisms simply need more iron for electron transport proteins in order to generate enough ATP for alginate synthesis. Interestingly, one of the genes also controlled by *P. aeruginosa* Fur, *nuoA*, encodes a subunit of complex I (NADH:ubiquinone reductase) of the electron transport chain (47). Similarly, elevated activity of FumC is probably a compensatory effect of decreased Fe-Fum activity (30). In this setting, reducing equivalents (NADH and reduced flavin adenine dinucleotide) provided by TCA cycle activity would still be funneled into the electron transport chain for the production of ATP, necessary for optimal performance of alginate biosynthetic machinery.

Another scenario comes into play when alginate is secreted from the bacterium. Alginate, being a polymer of negatively charged β-D-mannuronate and α-L-guluronate residues, may act in two ways once secreted from the cell. First, it could scavenge iron directly by binding of acidic groups to free or siderophore-complexed Fe^{3+}. This has been confirmed in physicochemical studies (for a review, see reference 24). Second, it may act as a physical barrier by limiting the accessibility of the *P. aeruginosa* siderophores ferripyochelin and ferripyoverdin (levels which are elevated in mucoid organisms [30]) to their respective cell surface receptors FptA and FpvA (1, 43), ultimately leading to an iron-limited state, similar to that of *P. aeruginosa fur* mutants (31).

In conclusion, it is likely that the *fagA-fumC-orfX-sodA* operon is transcribed in vivo since there is a selection for mucoidy and elevated siderophore levels in CF sputa (28). In addition, because the Fur/Fe^{2+} repressor is sensitive to redox changes, the increased transcription of the *fagA-fumC-orfX-sodA* operon (30) in mucoid bacteria suggests that the intracellular redox status (E_0) of these organisms is elevated in the CF airways. Because 30 to 40% of patients with CF are anemic relative to normal individuals (2), it is possible that this anomaly either contributes to the phenotypic switching from the nonmucoid to the mucoid state or, combined with alginate itself, simply provides a selective pressure for maintenance of mucoidy in vivo.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants AI-32085 (D.J.H.) and AI15690 (M.L.V.).

REFERENCES

1. **Ankenbauer, R. G., and H. N. Quan.** 1994. FptA, the Fe(III)-pyochelin receptor of *Pseudomonas aeruginosa*: a phenolate siderophore receptor ho-

mologous to hydroxamate siderophore receptors. J. Bacteriol. **176:**307–319.
2. **Ater, J. L., J. J. Herbst, S. A. Landaw, and R. T. O'Brien.** 1983. Relative anemia and iron deficiency in cystic fibrosis. Pedriatrics **71:**810–814.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. D. Seidman, and K. Struhl (ed.).** 1991. Current protocols in molecular biology. John Wiley & Sons, New York, N.Y.
4. **Bayer, A. S., F. Eftekhar, J. Tu, C. C. Nast, and D. P. Speert.** 1990. Oxygen-dependent up-regulation of mucoid exopolysaccharide (alginate) production in *Pseudomonas aeruginosa*. Infect. Immun. **58:**1344–1349.
5. **Baynham, P. J., and D. J. Wozniak.** 1996. Identification and characterization of AlgZ, an AlgT-dependent DNA-binding protein required for *Pseudomonas aeruginosa algD* transcription. Mol. Microbiol. **22:**97–108.
6. **Berry, A., J. D. DeVault, and A. M. Chakrabarty.** 1989. High osmolarity is a signal for enhanced *algD* transcription in mucoid and nonmucoid *Pseudomonas aeruginosa* strains. J. Bacteriol. **171:**2312–2317.
7. **Boat, T. F., M. J. Welsh, and A. L. Beaudet.** 1989. Cystic fibrosis, p. 2649–2680. *In* C. L. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle (ed.), The metabolic basis of inherited disease, vol. 1. McGraw-Hill, New York, N.Y.
8. **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.
9. **Calderwood, S. B., and J. J. Mekalanos.** 1987. Iron regulation of the Shiga-like toxin expression in *Escherichia coli* is mediated by the *fur* locus. J. Bacteriol. **169:**4759–4764.
10. **Chitnis, C. E., and D. E. Ohman.** 1990. Cloning of *Pseudomonas aeruginosa algG*, which controls alginate structure. J. Bacteriol. **172:**2894–2900.
11. **Chu, L., T. May, A. M. Chakrabarty, and T. Misra.** 1991. Nucleotide sequence and expression of the *algE* gene involved in alginate biosynthesis by *Pseudomonas aeruginosa*. Gene **107:**1–10.
12. **Clare, D. A., M. N. Duong, D. Darr, F. Archibald, and I. Fridovich.** 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.
13. **Compan, I., and D. Touati.** 1993. Interaction of six global transcription regulators in expression of manganese superoxide dismutase in *Escherichia coli* K-12. J. Bacteriol. **175:**1687–1696.
14. **de Lorenzo, V., F. Giovannini, M. Herrero, and J. B. Neilands.** 1988. Metal ion regulation of gene expression: Fur repressor-operator interaction at the promoter region of the aerobactin system of pColV-K30. J. Mol. Biol. **203:** 875–884.
14a. **Deretic, V.** Personal communication.
15. **Deretic, V., R. Dikshit, M. Konyecsni, A. M. Chakrabarty, and T. K. Misra.** 1989. The *algR* gene, which regulates mucoidy in *Pseudomonas aeruginosa*, belongs to a class of environmentally responsive genes. J. Bacteriol. **171:** 1278–1283.
16. **Deretic, V., J. F. Gill, and A. M. Chakrabarty.** 1987. Gene *algD* coding for GDPmannose dehydrogenase is transcriptionally activated in mucoid *Pseudomonas aeruginosa*. J. Bacteriol. **169:**351–358.
17. **Deretic, V., M. J. Schurr, J. C. Boucher, and D. W. Martin.** 1994. Conversion of *Pseudomonas aeruginosa* to mucoidy in cystic fibrosis: environmental stress and regulation of bacterial virulence by alternative sigma factors. J. Bacteriol. **176:**2773–2780.
18. **DeVault, J. D., K. Kimbara, and A. M. Chakrabarty.** 1990. Pulmonary dehydration and infection in cystic fibrosis: evidence that ethanol activates gene expression and induction of mucoidy in *Pseudomonas aeruginosa*. Mol. Microbiol. **4:**737–745.
19. **DeVries, C. A., and D. E. Ohman.** 1994. Mucoid-to-nonmucoid conversion in alginate-producing *Pseudomonas aeruginosa* often results from spontaneous mutations in *algT*, encoding a putative alternate sigma factor, and shows evidence for autoregulation. J. Bacteriol. **176:**6677–6687.
20. **Flynn, J. L., and D. E. Ohman.** 1988. Cloning of genes from mucoid *Pseudomonas aeruginosa* which control spontaneous conversion to the alginate production phenotype. J. Bacteriol. **170:**1452–1460.
21. **Flynn, J. L., and D. E. Ohman.** 1988. Use of a gene replacement cosmid vector for cloning alginate conversion genes from mucoid and nonmucoid *Pseudomonas aeruginosa* strains: *algS* controls expression of *algT*. J. Bacteriol. **170:**3228–3236.
22. **Franklin, M. J., and D. E. Ohman.** 1993. Identification of *algF* in the alginate biosynthetic gene cluster of *Pseudomonas aeruginosa* which is required for alginate acetylation. J. Bacteriol. **175:**5057–5065.
23. **Franklin, M. J., and D. E. Ohman.** 1996. Identification of *algI* and *algJ* in the *Pseudomonas aeruginosa* alginate biosynthetic gene cluster which are required for alginate O acetylation. J. Bacteriol. **178:**2186–2195.
24. **Gacesa, P.** 1988. Alginates. Carbohydr. Polym. **8:**161–182.
25. **Goldberg, J. B., W. L. Gorman, J. L. Flynn, and D. E. Ohman.** 1993. A mutation in *algN* permits trans activation of alginate production by *algT* in *Pseudomonas aeruginosa*. J. Bacteriol. **175:**1303–1308.
26. **Goldberg, J. B., and D. E. Ohman.** 1984. Cloning and expression in *Pseudomonas aeruginosa* of a gene involved in the production of alginate. J. Bacteriol. **158:**1115–1121.
27. **Govan, J. R. W., and V. Deretic.** 1996. Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Microbiol. Rev. **60:**539–574.
28. **Haas, B., E. Murphy, and D. Castignetti.** 1991. Siderophore synthesis by mucoid *Pseudomonas aeruginosa* strains isolated from cystic fibrosis patients. Can. J. Microbiol. **37:**654–657.
29. **Hantke, K.** 1987. Detection procedure for deregulated iron transport mutant (*fur*) in *Escherichia coli* K-12: fur not only affects iron metabolism. Mol. Gen. Genet. **210:**135–139.
29a. **Hassett, D. J.** Unpublished observations.
30. **Hassett, D. J., M. L. Howell, P. A. Sokol, M. L. Vasil, and G. E. Dean.** 1997. Fumarase C activity is elevated in response to iron deprivation and in mucoid, alginate-producing *Pseudomonas aeruginosa*: cloning and characterization of *fumC* and purification of native FumC. J. Bacteriol. **179:**1441–1450.
31. **Hassett, D. J., P. A. Sokol, U. A. Ochsner, M. L. Vasil, M. L. Howell, and J.-F. Ma.** 1996. Ferric uptake regulator (Fur) mutants of *Pseudomonas aeruginosa* demonstrate defective siderophore-mediated iron uptake and altered aerobic metabolism. J. Bacteriol. **178:**3996–4003.
32. **Hassett, D. J., W. A. Woodruff, D. J. Wozniak, M. L. Vasil, M. S. Cohen, and D. E. Ohman.** 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.
33. **Hill, R. L., and R. H. Bradshaw.** 1969. Fumarase. Methods Enzymol. **13:**91–99.
34. **Hiratsu, K., M. Amemura, H. Nashimoto, H. Shinagawa, and K. Makino.** 1995. The *rpoE* gene of *Escherichia coli*, which encodes σ^E, is essential for bacterial growth at high temperatures. J. Bacteriol. **177:**2918–2922.
35. **Holloway, B. W., V. Krishnapillai, and A. F. Morgan.** 1979. Chromosomal genetics of *Pseudomonas*. Microbiol. Rev. **43:**73–102.
36. **Kato, J., L. Chu, K. Kitano, J. D. DeVault, K. Kimbara, A. M. Chakrabarty, and T. K. Misra.** 1989. Nucleotide sequence of a regulatory region controlling alginate synthesis in *Pseudomonas aeruginosa*: characterization of the *algR2* gene. Gene **84:**31–38.
37. **Knutson, C. A., and A. Jeanes.** 1968. A new modification of the carbazole analysis: application to heteropolysaccharides. Anal. Biochem. **24:**470–481.
38. **Krieg, D. P., J. A. Bass, and S. J. Mattingly.** 1986. Aeration selects for mucoid phenotype of *Pseudomonas aeruginosa*. J. Bacteriol. **24:**986–990.
39. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
40. **Martin, D. W., B. W. Holloway, and V. Deretic.** 1993. Characterization of a locus determining the mucoid status of *Pseudomonas aeruginosa*: AlgU shows sequence similarities with a *Bacillus* sigma factor. J. Bacteriol. **175:** 1153–1164.
41. **Martin, D. W., M. J. Schurr, M. H. Mudd, and V. Deretic.** 1993. Mechanism of conversion to mucoidy in *Pseudomonas aeruginosa* infecting cystic fibrosis patients. Proc. Natl. Acad. Sci. USA **90:**8377–8381.
42. **Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12:**7035–7056.
43. **Meyer, J. M., D. Hohnadel, A. Khan, and P. Cornelis.** 1990. Pyoverdin-facilitated iron uptake in *Pseudomonas aeruginosa*: immunological characterization of the ferripyoverdin receptor. Mol. Microbiol. **4:**1401–1405.
44. **Mian, J. F., T. R. Jarman, and R. C. Righelato.** 1978. Biosynthesis of exopolysaccharide by *Pseudomonas aeruginosa*. J. Bacteriol. **134:**418–422.
45. **Mohr, C. D., D. W. Martin, W. M. Konyecsni, J. R. W. Govan, S. Lory, and V. Deretic.** 1990. Role of the far-upstream sites of the *algD* promoter and the *algR* and *rpoN* genes in environmental modulation of mucoidy in *Pseudomonas aeruginosa*. J. Bacteriol. **172:**6576–6580.
46. **Ochsner, U. A., A. I. Vasil, and M. L. Vasil.** 1995. Role of the ferric uptake regulator of *Pseudomonas aeruginosa* in the regulation of siderophores and exotoxin A expression: purification and activity on iron-regulated promoters. J. Bacteriol. **177:**7194–7201.
47. **Ochsner, U. A., and M. L. Vasil.** 1996. Gene regulation by the ferric uptake regulator (Fur) in *Pseudomonas aeruginosa*: cycle selection of iron-regulated genes. Proc. Natl. Acad. Sci. USA **93:**4409–4414.
47a. **Ohman, D.** Personal communication.
48. **Prince, R. W., C. D. Cox, and M. L. Vasil.** 1992. Molecular cloning and sequencing of the *Pseudomonas aeruginosa fur* gene: coordinate regulation of siderophore and exotoxin A production. J. Bacteriol. **175:**2589–2598.
49. **Prince, R. W., D. G. Storey, A. I. Vasil, and M. L. Vasil.** 1991. Regulation of *toxA* and *regA* by the *Escherichia coli fur* gene and identification of a Fur homologue in *Pseudomonas aeruginosa* PA103 and PAO1. Mol. Microbiol. **5:**2823–2831.
50. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
51. **Schurr, M. J., H. Yu, J. C. Boucher, N. S. Hibler, and V. Deretic.** 1995. Multiple promoters and induction by heat shock of the gene encoding the alternative sigma factor AlgU (s^E) which controls mucoidy in cystic fibrosis

isolates of *Pseudomonas aeruginosa*. J. Bacteriol. **177:**5670–5679.
52. **Schurr, M. J., H. Yu, J. M. Martinez-Salazar, J. C. Boucher, and V. Deretic.** 1996. Control of AlgU, a member of the s^E-like family of stress sigma factors, by the negative regulators MucA and MucB and *Pseudomonas aeruginosa* conversion to mucoidy in cystic fibrosis. J. Bacteriol. **178:**4997–5004.
53. **Schweizer, H. P.** 1992. Allelic exchange in *Pseudomonas aeruginosa* using novel ColE1-type vectors and a family of cassettes containing a portable *oriT* and the counter-selectable *Bacillus subtilis sacB* marker. Mol. Microbiol. **6:**1195–1204.
54. **Schweizer, H. P.** 1993. Small broad-host-range gentamycin resistance gene cassettes for site-specific insertion and deletion mutagenesis. BioTechniques **15:**831–833.
55. **Schwyn, B., and J. B. Neilands.** 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. **160:**47–56.
56. **Silver, S., P. Johnseine, E. Whitney, and D. Clark.** 1972. Manganese-resistant mutants of *Escherichia coli*: physiological and genetics studies. J. Bacteriol. **110:**186–195.
57. **Simon, R., U. Priefer, and A. Puehller.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1:**784–791.
58. **Tardat, B., and D. Touati.** 1993. Iron and oxygen regulation of *Escherichia coli* MnSOD expression: competition between the global regulators Fur and ArcA for binding to DNA. Mol. Microbiol. **9:**53–63.
59. **Terry, J. M., S. E. Pina, and S. J. Mattingly.** 1991. Environmental conditions which influence mucoid conversion in *Pseudomonas aeruginosa* PAO1. Infect. Immun. **59:**471–477.
60. **Terry, J. M., S. E. Pina, and S. J. Mattingly.** 1992. Role of energy metabolism in conversion of nonmucoid *Pseudomonas aeruginosa* to the mucoid phenotype. Infect. Immun. **60:**1329–1335.
61. **Weast, R. C. E.** 1969. Handbook of chemistry and physics. The Chemical Rubber Co., Cleveland, Ohio.
62. **Wozniak, D. J., and D. E. Ohman.** 1993. Involvement of the alginate *algT* gene and integration host factor in the regulation of the *Pseudomonas aeruginosa algB* gene. J. Bacteriol. **175:**4145–4153.
63. **Wozniak, D. J., and D. E. Ohman.** 1994. Transcriptional analysis of the *Pseudomonas aeruginosa* genes *algR*, *algB*, and *algD* reveals a hierarchy of alginate gene expression which is modulated by *algT*. J. Bacteriol. **176:**6007–6014.
64. **Xie, Z.-D., C. D. Hershberger, S. Shankar, R. W. Ye, and A. M. Chakrabarty.** 1996. Sigma factor-anti sigma factor interaction in alginate synthesis: inhibition of AlgT by MucA. J. Bacteriol. **178:**4990–4996.
65. **Yu, H., J. C. Boucher, N. S. Hibler, and V. Deretic.** 1996. Virulence properties of *Pseudomonas aeruginosa* lacking the extreme-stress sigma factor AlgU (σ^E). Infect. Immun. **64:**2774–2781.
66. **Zielinski, N., A. Chakrabarty, and A. Berry.** 1991. Characterization and regulation of the *Pseudomonas aeruginosa algC* gene encoding phosphomannomutase. J. Biol. Chem. **266:**9754–9763.