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Coordinate Regulation of Siderophore and Exotoxin A Production: Molecular Cloning and Sequencing of the *Pseudomonas aeruginosa fur* Gene

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A 5.9-kb DNA fragment was cloned from *Pseudomonas aeruginosa* PA103 by its ability to functionally complement a *fur* mutation in *Escherichia coli*. A *fur* null mutant *E. coli* strain that contains multiple copies of the 5.9-kb DNA fragment produces a 15-kDa protein which cross-reacts with a polyclonal anti-*E. coli* Fur serum. Sequencing of a subclone of the 5.9-kb DNA fragment identified an open reading frame predicted to encode a protein 53% identical to *E. coli* Fur and 49% identical to *Vibrio cholerae* Fur and *Yersinia pestis* Fur. While there is extensive homology among these Fur proteins, Fur from *P. aeruginosa* differs markedly at its carboxy terminus from all of the other Fur proteins. It has been proposed that this region is a metal-binding domain in *E. coli* Fur. A positive selection procedure involving the isolation of manganese-resistant mutants was used to isolate mutants of strain PA103 that produce altered Fur proteins. These manganese-resistant Fur mutants constitutively produce siderophores and exotoxin A when grown in concentrations of iron that normally repress their production. A multicopy plasmid carrying the *P. aeruginosa fur* gene restores manganese susceptibility and wild-type regulation of exotoxin A and siderophore production in these Fur mutants.

Production of *Pseudomonas aeruginosa* exotoxin A is regulated by the amount of iron in the growth environment (6). Transcription of the gene encoding exotoxin A (*toxA*) is repressed when the concentration of iron is high and derepressed when the concentration of iron is low (9, 19, 22, 27, 32, 53). The mechanism governing the regulation of *toxA* by iron is not completely understood, but it is likely that most iron regulation occurs through the *regAB* operon (19, 20, 27, 52).

The *regAB* operon, originally cloned by Hedstrom et al. (25) and called *toxR*, is a positive activator of *toxA* transcription. This operon is regulated by iron at the transcriptional level, analogous to *toxA* (19). How the *regAB* genes are regulated by iron is not understood, but the mechanism appears to be complex. Transcription of the *regAB* operon is controlled by two promoters (19). Transcription from the P1 promoter occurs early in the growth cycle and is not tightly iron regulated (19, 48, 49), whereas transcription from the P2 promoter occurs late in the growth cycle and is tightly iron regulated (19, 48, 49).

Similar to other gram-negative bacteria, *P. aeruginosa* possesses a high-affinity iron uptake system which is repressed by high iron concentrations and derepressed in low-iron environments (4, 11). However, little is known about the regulation of this high-affinity uptake system. In *Escherichia coli* high-affinity iron uptake systems are controlled by the *E. coli fur* (ferric uptake regulator) gene. *E. coli* Fur uses iron (Fe^{2+}) as a corepressor, and this complex binds to a consensus sequence (Fur box) found in the promoter regions of many iron-regulated genes. Binding of Fur to the operator blocks transcription of these genes (3, 7, 12, 14). Recently, we reported that multiple copies of *E. coli fur* in *P. aeruginosa* PA103 could regulate the *toxA* and *regAB* genes (38). Evidence was also presented that a Fur homolog exists in *P. aeruginosa* PAO1 and PA103.

In this report, we describe the cloning and characterization of a *fur* homolog from *P. aeruginosa* and demonstrate that this gene regulates the production of exotoxin A and siderophores in *P. aeruginosa*. A positive selection method for isolating mutants of *P. aeruginosa* that produce altered Fur proteins is described.

MATERIALS AND METHODS

Bacterial strains, plasmids, and medium. The bacterial strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* strains used for the analysis of exotoxin A or for β-galactosidase assays were grown in low-iron Trypticase soy broth dialysate (DTSB) (35) or DTSB supplemented with 36 μM $FeSO_4$ at 32°C as previously described (38). *E. coli* strains used for β-galactosidase assays were grown in DTSB with 36 μM iron or without added iron at 37°C. *E. coli* strains used to make whole-cell extracts for the analysis of a cross-reactive Fur protein were grown overnight in brain heart infusion medium (Difco Laboratories) at 37°C. *P. aeruginosa* strains used to make whole-cell extracts for the analysis of *P. aeruginosa* Fur were grown overnight at 32°C in 0.5% Bacto-tryptone (Difco)–57 mM K_2SO_4–6.9 mM $MgCl_2$. The selection medium and growth conditions for the isolation of manganese-resistant mutants were as described previously (44). Cultures to determine siderophore production were grown overnight in 1% Casamino Acids with shaking at 37°C, washed in sterile distilled water, and inoculated at 10^3 CFU/ml into 5-ml quantities of 1% Casamino Acids containing concentrations of iron ranging from 0 to 200 μM. When needed, 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-Gal) was used at a concentration of 40 mg/liter.

Antibiotics were used at the following concentrations: for

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Reference or source
E. coli		
1618	*thr lac ser fhuA rpsL thi hsdR fiu*::MudI(Ap *lac*) *fur-31*	23
SBC24	Δ(*ara-leu*)*7697 araD139* Δ(*lac*)*X74 galE galK rpsL thi malF*Δ*3 phoA* Δ*Pvu*II *phoR zad*::Tn*10 pcnB*/F′ *lac pro lacI*^{q} *fur*::Tn*5*	7
P. aeruginosa		
PA103	Prototroph, hypertoxigenic strain	51
PA103M	Fluorescent PA103, manganese resistant, constitutive pyoverdin production	This study
PA103MC	PA103M with a *tox4*::*lacZ* fusion integrated into its chromosome	This study
Plasmids		
pACYC184	Cm^r Tc^r	8
pMMB207	Cm^r	34
pAC5.9	Cm^r	This study
pMM9.0	Cm^r	This study
pBluescript SK(+)	Ap^r; phagemid	Stratagene
RSF1010	Sm^r Su^r	2
pUCRSF	Ap^r Sm^r Su^r; hybrid of pUC18 and RSF1010	52
pEHFUR	1.8-kb *Eco*RI-*Hin*dIII DNA fragment containing *P. aeruginosa fur* in pUCRSF	This study
pKT230	Hybrid of RSF1010 and pACYC177; Km^r Sm^r	2
pKTFUR	3.1-kb *Hin*dIII DNA fragment containing *P. aeruginosa fur* cloned into pKT230; Sm^r	This study

[a] The genotype symbols have been described by Bachmann (1).

E. coli, 50 μg of carbenicillin per ml, 50 μg of chloramphenicol per ml, 50 μg of streptomycin per ml, and 10 μg of tetracycline per ml; for *P. aeruginosa*, 500 μg of carbenicillin per ml and 500 μg of streptomycin per ml.

Assay of exotoxin A. Detection of exotoxin A in culture supernatants by Western blotting (immunoblotting) was as previously described (52).

Detection of cross-reactive Fur protein. Whole-cell extracts were made as described by Sambrook et al. (40). Twelve microliters of extract for each strain was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred to Nitroplus paper (MSI) by using a Bio-Rad (Richmond, Calif.) semi-dry blotting apparatus. The blotted proteins were screened with a polyclonal rabbit anti-*E. coli* Fur serum at a dilution of 1:500 and detected with a Promega (Madison, Wis.) alkaline phosphatase (ProtoBlot) kit.

β-Galactosidase assays. β-Galactosidase activity was assayed and calculated as described by Miller and is defined as units per unit of optical density at 600 nm (33).

Electroporation. Electroporation of *E. coli* 1618 with a Gene Pulser and Pulse Controller (Bio-Rad Laboratories) was done by the method of Dower et al. (16).

DNA manipulations. The techniques used for molecular cloning were as described previously (40). Restriction enzymes, T4 ligase, and calf intestinal phosphatase were obtained from Bethesda Research Laboratories (Gaithersburg, Md.) and used as directed by the supplier. *P. aeruginosa* chromosomal DNA was isolated as previously described (51). PA103 chromosomal DNA used to make gene banks in *E. coli* 1618 was digested at 37°C for 30 min with *Sau*3A1 at an enzyme concentration that was predetermined to give a majority of fragments in the range of 5.0 to 9.0 kb. After digestion, the DNA was extracted with phenol chloroform and ethanol precipitated. This DNA was then ligated into plasmid pACYC184 or pMMB207. Transformation of *E. coli* was done by the method of Kushner (30). Transfer of plasmids from *E. coli* to *P. aeruginosa* by triparental matings with plasmid pRK2013 was as previously described (17).

Determination of pyoverdin concentrations. Pyoverdin was measured by diluting overnight cultures into 10 mM Tris-hydrochloride buffer (pH 7.5) and measuring the fluorescence emission at 460 nm during excitation at 400 nm.

Sequencing. A 3.1-kb *Hin*dIII DNA fragment from plasmid pAC5.9 was cloned in both orientations into the phagemid pBluescript SK(+) (Stratagene). Single-stranded DNA was made by using helper phage and sequenced according to the method of Sanger et al. (41), using a Sequenase 2.0 kit (United States Biochemical). Oligonucleotide primers were synthesized as needed to sequence across both strands of the cloned fragment.

Nucleotide sequence accession number. The sequence reported for *P. aeruginosa fur* has been entered into GenBank under accession number L00604.

RESULTS

Cloning of a *fur* homolog from *P. aeruginosa* PA103. PA103 chromosomal DNA was partially digested with *Sau*3A1 as described in Materials and Methods to produce a majority of DNA fragments in the size range of 5.0 to 9.0 kb. These fragments were ligated into plasmids pACYC184 (8) and MMB207 (34), which had been digested with *Bam*HI. The ligated products were then electroporated into *E. coli* 1618. *E. coli* 1618 has an undefined *fur* mutation and also contains a *lacZ* fusion in the iron-regulated gene *fiu* (23). When strain 1618 is plated onto medium that contains a high iron concentration (DTSB plus 100 μM Fe_2SO_4) and X-Gal, the colonies are dark blue. It was hypothesized that if the gene banks contained the gene encoding the *fur* homolog, this gene would complement the *fur* mutation in strain 1618. Therefore, colonies that contained this gene would be white or light blue on high-iron medium that contained X-Gal. With this strategy, 7 and 3 clones (of approximately 30,000) that were light blue on high-iron plates were found in the pACYC184 and MMB207 banks, respectively. Plasmid DNA was isolated from these clones and used to retransform strain 1618. Upon retransformation, three of the seven isolated plasmids from the pACYC184 gene bank and two of the three isolated plasmids from the MMB207 gene bank were still able to repress the constitutive expression of *fiu* in strain 1618. Restriction enzyme analysis determined that the

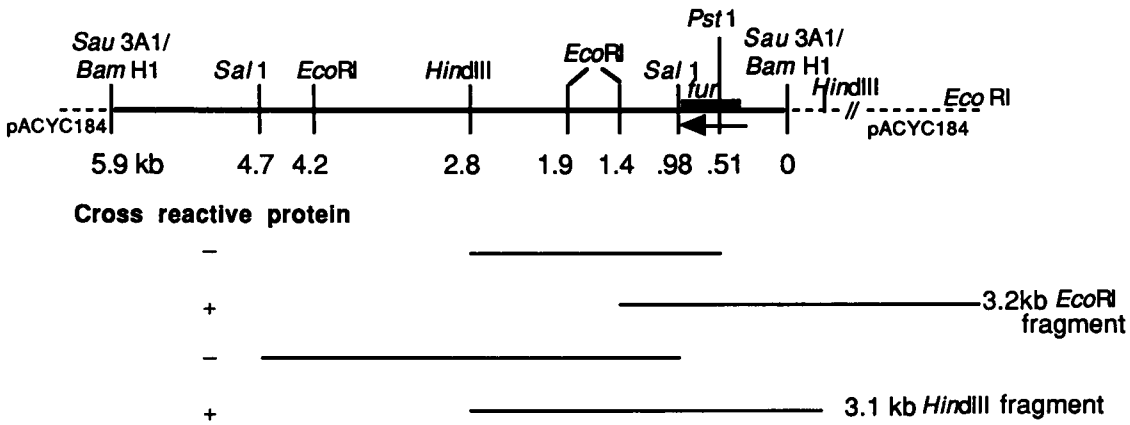

FIG. 1. Restriction map of the 5.9-kb *Sau*3A1 *P. aeruginosa* DNA fragment cloned into the *Bam*HI site of pACYC184. The lower portion indicates which subclones of the 5.9-kb fragment can still produce a protein that cross-reacts with an anti-*E. coli* Fur serum by Western blot analysis. The thick black line shows the relative position of the *P. aeruginosa fur* gene. Relevant restriction sites are shown.

three clones from the pACYC184 bank contained the same 5.9-kb DNA insert (Fig. 1) and that the two clones from the MMB207 bank contained the same 9.0-kb DNA fragment (data not shown). The restriction map of the 9.0-kb DNA fragment is distinct from that of the 5.9-kb DNA fragment (data not shown).

Table 2 shows that β-galactosidase production in strain 1618 containing multiple copies of the 5.9-kb DNA fragment (pAC5.9) was repressed in both high- and low-iron media, although not as markedly in low-iron medium. In contrast, β-galactosidase production in strain 1618 containing multiple copies of the 9.0-kb DNA fragment (pMM9.0) was repressed to the same degree (sixfold) in both iron concentrations (Table 2). Strain 1618 containing multiple copies of a plasmid carrying *E. coli fur* (pMH1) had β-galactosidase activities that were repressed in both high- and low-iron media. Strain 1618 containing the vector controls (pACYC184 or MMB207) had β-galactosidase activities that were constitutive in both high- and low-iron media.

It has been reported that *E. coli* strains which have *fur* mutations are unable to grow on minimal medium containing succinate as the sole carbon source (4, 24). Therefore, it was of interest to determine if either the 5.9-kb DNA fragment or the 9.0-kb DNA fragment contained a gene or genes that would enable the *fur* mutant *E. coli* strain 1618 to grow on minimal medium containing succinate as the sole carbon source. *E. coli* 1618 containing multiple copies of pAC5.9 or pMM9.0 grew on minimal medium plates containing succinate as the sole carbon source (Table 2).

Western blot analysis of SBC24 containing the 5.9-kb fragment and the 9.0-kb fragment. Western blot analysis was used to determine if either the 5.9-kb DNA fragment or the 9.0-kb DNA fragment contains the gene that encodes the cross-reactive Fur protein. pAC5.9 and pMM9.0 were used to transform *E. coli* SBC24, a *fur* null mutant (7). Western blot analysis of whole-cell extracts from strain SBC24 (pAC5.9) contained a protein which cross-reacts with an anti-*E. coli* Fur serum. SBC24 containing a vector control did not produce a cross-reactive Fur protein (Fig. 2). Western blot analysis of whole-cell extracts of SBC24(pMM9.0) with the same antiserum indicated that the 9.0-kb fragment of DNA does not encode a similar cross-reactive Fur protein (data not shown). Additional data regarding the 9.0-kb DNA clone are not included in this report.

Sequencing of the cloned 5.9-kb DNA fragment. A partial restriction map of the 5.9-kb *Sau*3A1 DNA fragment that was cloned into the *Bam*HI site of pACYC184 is shown in Fig. 1. Subclones were generated by using restriction endonuclease enzymes *Hin*dIII, *Sal*I, *Pst*I, and *Eco*RI (Fig. 1). These fragments were cloned into the phagemid vector pBluescript SK(+) in both orientations, and the ligated

TABLE 2. β-Galactosidase production in *E. coli* 1618 containing potential *P. aeruginosa fur* clones

Strain	U[a]		Growth on succinate
	With Fe	Without Fe	
1618(pACYC184)	681	703	–
1618(MMB207)	720	732	–
1618(MM9.0)	102	103	+
1618(pMH1)	2.6	11.2	+
1618(pAC5.9)	4.3	117	+

[a] β-Galactosidase activity is expressed as units per unit of optical density at 600 nm as defined by Miller (33).

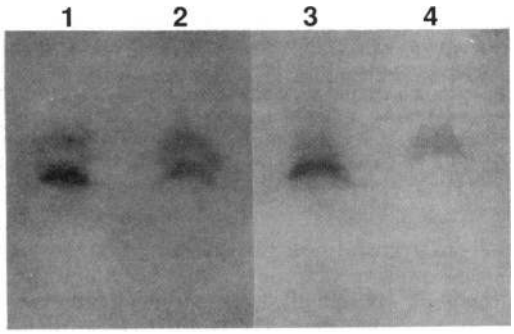

FIG. 2. Western blot analysis demonstrating that a 5.9-kb DNA fragment cloned from *P. aeruginosa* PA103 carries a gene which encodes a cross-reactive Fur protein. Whole-cell extracts were made from the following cultures, which were grown overnight in brain heart infusion medium: SBC24(pMH1) (lane 1), SBC24 (pAC5.9) (lane 2), PA103 (lane 3), and SBC24(pACYC184) (lane 4). Cross-reactive protein was detected by screening the blot with a polyclonal rabbit anti-*E. coli* Fur serum. The upper band seen in all lanes is nonspecific binding.

A

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1 AAACATCCCG CGCTGAGAAA GCAGAC  ATG GTT GAA AAT AGC GAA CTT CGA AAA
                    SD          Met Val Glu Asn Ser Glu Leu Arg Lys

54   GCC GGC CTT AAA GTG ACC CTG CCG CGG GTC AAG ATC CTG CAG ATG CTC GAC
     Ala Gly Leu Lys Val Thr Leu Pro Arg Val Lys Ile Leu Gln Met Leu Asp

105  TCG GCC GAG CAA CGC CAC ATG AGC GCC GAA GAC GTG TAC AAG GCG CTG ATG
     Ser Ala Glu Gln Arg His Met Ser Ala Glu Asp Val Tyr Lys Ala Leu Met

156  GAA GCA GGC GAG GAC GTG GGC CTG GCA ACC GTC TAT CGG GTG CTG ACC CAG
     Glu Ala Gly Glu Asp Val Gly Leu Ala Thr Val Tyr Arg Val Leu Thr Gln

207  TTC GAG GCC GCC GGC CTG GTG GTG CGT CAC AAC TTC GAT GGC GGC CAT GCC
     Phe Glu Ala Ala Gly Leu Val Val Arg His Asn Phe Asp Gly Gly His Ala

258  GTG TTC GAG CTC GCC GAT AGC GGC CAC CAC GAC CAC ATG GTC TGC GTC GAT
     Val Phe Glu Leu Ala Asp Ser Gly His His Asp His Met Val Cys Val Asp

309  ACC GGC GAG GTG ATC GAG TTC ATG GAC GCG GAA ATC GAG AAG CGC CAG AAG
     Thr Gly Glu Val Ile Glu Phe Met Asp Ala Glu Ile Glu Lys Arg Gln Lys

360  GAA ATC GTC CGC GAG CGC GGC TTC GAG CTG GTC GAT CAC AAT CTG GTG CTC
     Glu Ile Val Arg Glu Arg Gly Phe Glu Leu Val Asp His Asn Leu Val Leu

411  TAC GTG CGC AAG AAG AAG TAG
     Tyr Val Arg Lys Lys Lys !!!

432  TCGCGCAACGGTACCATGCGAAAGCGGCGACCCTGGGGGTCGCCGTTTTCATTTT
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B

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                10         20         30         40          50
YPFUR    MTDNNKALKNAGLKVTLPRLKILEVLQNPACHHVSAEDLYKILIDI--GEEI
VCFUR    MSDNNQALKDAGLKVTLPRLKILEVLQQPECQHISAEELYKKLIDL--SEEI
ECFUR    MTDNNTALKKAGLKVTLPRLKILEVLQEPDNHHVSAEDLYKRLIDM--GEEI
PAFUR    MVENSE-LRKAGLKVTLPRVKILQMLDSAEQRHMSAEDVYKAL--MEAGEDV
         *-**-*********************-**************-*  *  ****

                60         70         80         90         100
YPFUR    GLATVYRCSEQFDDAGIVTRHNFEGGKSVFELTQQHHHDHLICLDCGKVI
VCFUR    GLATVYRVLNQFDDAGIVTRHHFEGGKSVFELSTQHHHDHLVCLDCGEVI
ECFUR    GLATVYRVLNQFDDAGIVTRHNFEGGKSVFELTQQHHHDHLICLDCGKVI
PAFUR    GLATVYRVLTQFEAAGLVVRHNFDGGHAVFELADSGHHDHMVCVDTGEVI
         *********-***-*****-****************************-****

                110        120        130        140        150
YPFUR    EFSNESIESLQREIAKQH--GIKLTNHSLYLYGHCET-GNCREDESAHSKR.
VCFUR    EFSDDVIEQRQKEIAAKYNVQ--LTNHSLYLYGKCGSDGSCKDNPNAHKPKK
ECFUR    EFSDDSIEARQREIAAKH--GIRLTNHSLYLYGHCAE-GDCREDEHAHEGK.
PAFUR    EFMDAEIEKRQKEIVRERGFELVDHNLVLYVRKKK.................
         **-*---**-**********--**---*--****-*-
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FIG. 3. (A) Nucleotide and predicted amino acid sequences of *P. aeruginosa fur*. A putative Shine-Dalgarno (SD) sequence is underlined, and a potential transcriptional termination signal is double underlined. (B) Multiple alignment of the deduced amino acid sequences of Fur from *P. aeruginosa* (PAFUR), *E. coli* (ECFUR), *V. cholerae* (VCFUR), and *Y. pestis* (YPFUR). Below each amino acid of *P. aeruginosa* Fur, an asterisk indicates that the amino acid is identical or similar to an amino acid in *E. coli*, *V. cholerae*, or *Y. pestis* Fur at that position, while a dash indicates that the amino acid is neither identical nor similar to any amino acid at the corresponding position in any of the other three proteins. Shown in bold letters are the residues (mostly Cys and His) that are found in *E. coli*, *V. cholerae*, and *Y. pestis* Fur, but not *P. aeruginosa* Fur, that are thought to be critical to metal binding or a DNA-binding domain (see text). This multiple alignment was done by using the multiple sequence alignment function in the Hitachi DNASIS version 2.0 software for Macintosh computers.

products were transformed into strain SBC24. Western blot analysis was used to determine what region of the 5.9-kb DNA fragment contained the gene encoding the cross-reactive Fur protein. The 3.1-kb *Hin*dIII DNA fragment and the 3.2-kb *Eco*RI DNA fragment contained the gene encoding the cross-reactive Fur protein (Fig. 1). Production of this cross-reactive Fur protein by the *Hin*dIII fragment and the 3.2-kb *Eco*RI fragment was not orientation specific in pBluescript SK(+), suggesting that the cross-reactive protein could be expressed from its own promoter. A portion (905 bp) of the 3.1-kb *Hin*dIII DNA fragment (Fig. 1) was sequenced. This DNA fragment contained an open reading frame predicted to encode a protein with a high degree of homology to *E. coli* Fur (Fig. 1). The DNA sequence and predicted amino acid sequence of this gene, *P. aeruginosa fur*, are shown in Fig. 3. A comparison of the *P. aeruginosa fur* gene with *Vibrio cholerae fur* and *E. coli fur* indicates that *P. aeruginosa fur* is 55% identical to *V. cholerae fur* and 54% identical to *E. coli fur*. At the amino acid level, *P. aeruginosa* Fur is 53% identical to *E. coli* Fur and 49% identical to Fur from *V. cholerae* or *Yersinia pestis* (Fig. 3B). Overall, the Fur proteins from all of these organisms are >70% similar (Fig. 3B). A striking difference among the three Fur proteins is found at their carboxy-terminal ends. *P.*

aeruginosa Fur lacks the Cys-X_4-Cys-X_4-His-X-His sequence found in *E. coli* Fur and a similar sequence in *V. cholerae* or *Y. pestis* Fur (Fig. 3B) that has been proposed to be involved in metal binding (26). This sequence also resembles a DNA-binding motif (zinc finger) found in some eucaryotic DNA-binding proteins (28). In *P. aeruginosa* Fur, this sequence is replaced by Arg-Lys-Lys-Lys. It is also worth noting that *P. aeruginosa* Fur contains only one Cys residue, in contrast to the other Fur proteins, which have at least four. For the following reasons, it is unlikely that a sequencing error that would add amino acid sequences onto the C-terminal end of *P. aeruginosa* Fur was made: (i) there is a potential Rho-independent terminator located close to the predicted translational stop site, and (ii) an analysis of the DNA sequence (using codon preference) predicted for *P. aeruginosa* indicated that the translational stop site is located in the correct position.

Because *E. coli fur* has been shown to be autoregulated and regulated by the catabolite activator protein, the upstream region of *P. aeruginosa fur* was searched for sequences that resemble the consensus Fur-binding site and the consensus catabolite activator protein-binding site (13). No sequences that resembled either a consensus Fur-binding site or a consensus catabolite activator protein-binding site were found.

Effect of multiple copies of *P. aeruginosa fur* on expression of *toxA* in strain PA103C. In a previous study, we found that multiple copies of *E. coli fur* in strain PA103C resulted in the inhibition of *toxA* expression in both high- and low-iron media (38). Therefore, we wanted to determine if multiple copies of *P. aeruginosa fur* would have the same effect on *toxA* expression as multiple copies of *E. coli fur*. The 3.1-kb *Hin*dIII DNA fragment containing *P. aeruginosa fur* was isolated from plasmid pAC5.9 and cloned into the broad-host-range plasmid pKT230. The resultant plasmid, called pKTFUR, was conjugated into *P. aeruginosa* PA103C by triparental matings. PA103C is a derivative of strain PA103 that contains a *toxA*::*lacZ* fusion integrated into its chromosome at the *toxA* locus (52). β-Galactosidase activities of PA103C containing multiple copies of pKTFUR grown in high- and low-iron media, surprisingly, showed normal iron regulation (Fig. 4A). Since it was possible that *P. aeruginosa* Fur was not being produced from plasmid pKTFUR, Western blot analysis was used to determine if PA103C containing plasmid pKTFUR produced greater levels of Fur than PA103C did. This analysis indicated that Fur was being produced from pKTFUR (data not shown).

To be certain that multiple copies of *P. aeruginosa fur* have no effect on exotoxin A production in strain PA103, a DNA fragment containing *P. aeruginosa fur* but smaller than the one in pKTFUR was used. A 1.8-kb *Eco*RI-*Hin*dIII DNA fragment containing *P. aeruginosa fur* (Fig. 1) was cloned into pUC18. The broad-host-range plasmid RSF1010 was cloned into this plasmid at its unique *Eco*RI site to enable it to replicate in *P. aeruginosa*. The resultant plasmid, called pEHFUR, was mated into *P. aeruginosa* PA103. Western blot analysis for exotoxin A production was done with culture supernatants obtained from PA103 containing pEHFUR or a vector control (pUCRSF) from cultures grown in high- and low-iron media. This type of analysis again indicated that exotoxin A production was not affected by multiple copies of *P. aeruginosa fur* (Fig. 4B). Moreover, iron-regulated siderophore production in PA103 carrying multiple copies of pEHFUR was not altered (data not shown).

Positive selection of Fur mutants of *P. aeruginosa* by manganese selection. To better determine what function *P. aeruginosa fur* has and to determine if it regulates *toxA*, an attempt was made to construct a *fur* null mutant. For reasons that are unclear, it was not possible to make a *fur* mutant of strain PA103 by standard genetic techniques such as gene replacement or gene interruption. These methods have been used previously by this laboratory to construct mutants of the phospholipase C genes of *P. aeruginosa* PAO1 and the *toxA* gene of strain PA103 and PAO1, as well as to delete the entire *regAB* operon in PA103 (36, 37; data not shown). Fur mutants of *P. aeruginosa* PA103 were made, however, by a positive selection procedure described by Hantke (24). Hantke used this method to isolate *fur* mutants of *E. coli*, *Serratia* spp., and *Klebsiella* spp. Hantke also used this technique for *P. aeruginosa* but was unable to determine if it resulted in the selection of a *fur* mutant (24). A 100-μl portion of an overnight culture of strain PA103 (about 10^8

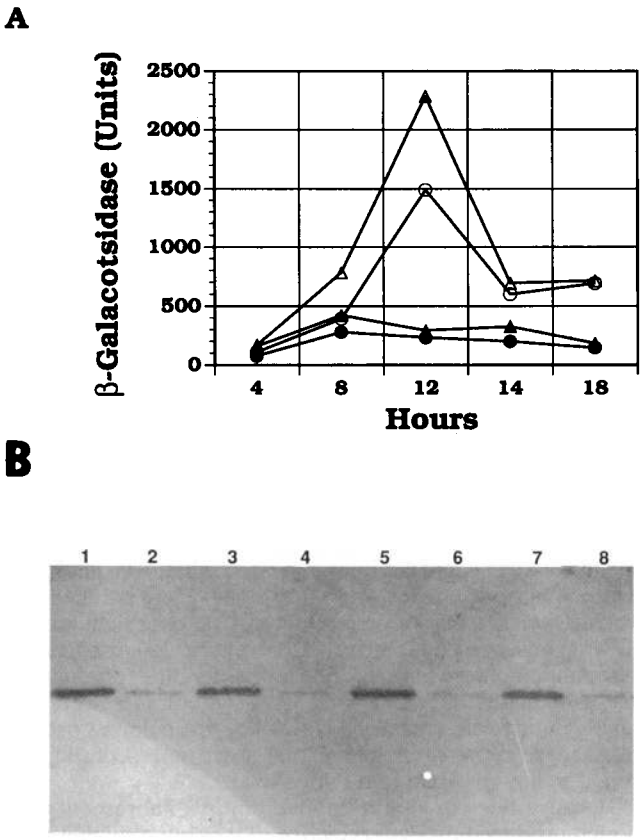

FIG. 4. (A) β-Galactosidase activities for PA103C(pKTFUR) (triangles) and PA103C(pKT230) (circles). Open symbols represent cultures that were grown in iron-deficient medium (DTSB), and closed symbols represent cultures that were grown in high-iron medium (DTSB with 36 μM iron). β-Galactosidase activity is reported as units per unit of optical density at 600 nm. Standard deviations were within 10% of the mean. (B) Western blot analysis of exotoxin A production by PA103(pEHFUR) (lanes 1, 2, 5, and 6) and PA103(pUCRSF) (lanes 3, 4, 7, and 8). Cultures were grown in DTSB for 16 h (lanes 1 through 4) or 18 h (lanes 5 through 8), and culture supernatants were assayed for exotoxin A by Western blotting with an affinity-purified anti-exotoxin A rabbit serum. Odd-numbered lanes represent cultures that were grown in iron-deficient medium (DTSB), and even-numbered lanes represent cultures that were grown in iron-rich medium (DTSB plus 36 μM iron).

TABLE 3. Pyoverdin concentrations in 1% Casamino Acids medium following growth at 37°C for 24 h with shaking

Fe concn (μM)	Pyoverdin concn (μg/ml) produced by strain:			
	PA103	PA103M	PA103M (pKT230)	PA103M (pKTFUR)
200	0	80.0	48.5	0
100	0	80.0	443.7	0
50	7.3	232.8	106.7	0
25	7.3	113.9	128.5	0
12.5	58.2	215.8	310.3	58.1
6.25	312.8	208.5	426.7	184.3
3.13	407.3	341.9	402.5	128.5
1.58	293.4	429.1	387.9	329.7
0.78	419.4	395.2	337.0	378.2
0	162.4	249.7	305.5	111.5

cells) was plated onto dilute tryptone agar plates that contained 10 mM $MnCl_2$ (44). After growth at 37°C for 24 h, manganese-resistant colonies appeared (about 200 per plate). These manganese-resistant PA103 clones were patched onto *Pseudomonas* isolation agar (Difco). After overnight growth, it was observed that about 1 of every 100 manganese-resistant colonies was secreting a bright yellow product into the agar plates. Under UV light, these colonies were also highly fluorescent compared with wild-type colonies or other manganese-resistant colonies that were not secreting the bright yellow product (data not shown).

Because the siderophore pyoverdin is a fluorescent compound produced by *P. aeruginosa*, it was possible that these fluorescent strains were producing pyoverdin constitutively. The production of pyoverdin by one of the fluorescent manganese-resistant strains (PA103M) was quantified by fluorescence emission at 460 nm (10) (Table 3). This analysis indicated that PA103M produces pyoverdin at iron concentrations (100 to 200 μM) that normally inhibit production in PA103. It should be noted that although PA103M has levels of pyoverdin only in the 80-μg/ml range, at the higher iron concentrations (100 to 200 μM), the levels of pyoverdin may actually be even higher. When PA103M and PA103M (KT230) are grown in media containing high iron concentrations (>36 μM), they produce a deep brown pigment in the culture medium. This pigment is caused by the formation of ferripyoverdin. Unfortunately, ferripyoverdin quenches the fluorescence of pyoverdin in this type of analysis, indicating that at high iron concentrations pyoverdin production by PA103M and PA103M(KT230) may actually be higher than those reported in Table 3.

To confirm that PA103M was producing pyoverdin constitutively, a rapid plate assay for siderophore production was used. PA103M was examined on Chrome Azurol blue agar plates as originally described by Schwyn and Neilands (43). In this assay, siderophores produced by bacteria growing on the plates remove the iron complexed to the highly colored dye contained in the plates. This removal of the iron from the dye is observed as a change in color of the dye. When PA103M was examined on these plates, it produced a siderophore(s) even on plates that contained an additional 100 μM $FeCl_3$ (data not shown).

Since *P. aeruginosa* produces two siderophores, pyoverdin and pyochelin, it is possible that PA103M also produces pyochelin constitutively. Therefore, the production of pyochelin by PA103M was quantified by thin-layer chromatography (10). This analysis indicated that pyochelin is produced at iron concentrations which normally repress its production in wild-type PA103 (data not shown).

Because siderophore production in PA103M was no longer regulated by iron, it was possible that other iron-regulated genes, such as *toxA*, were now also deregulated in this mutant. Cultures of PA103M were grown in low-iron or iron-rich medium, and the production of exotoxin A was examined by Western blotting with an affinity-purified anti-exotoxin serum (Fig. 5A). Exotoxin A production was constitutive in strain PA103M as compared with the parental strain PA103. Although only one time point is shown in Fig. 5A, the same result was obtained for cultures grown for 16 and 18 h in iron-rich and iron-deficient media (data not shown).

Analysis of Fur in independently isolated manganese-resistant mutants. Whole-cell extracts from three independently isolated manganese-resistant mutants of PA103 were analyzed by Western blotting with the anti-Fur serum. These strains produce a cross-reactive protein that migrates faster than *P. aeruginosa* Fur from the wild-type strain, which has an apparent size of 15 kDa when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 5B). One of these mutant strains, PA103M, appears to make a Fur protein that reproducibly migrates slightly faster than the wild type (Fig. 5B, lane 2). The other two manganese-resistant strains make Fur proteins which migrate considerably faster than wild-type Fur. The estimated molecular mass for these faster-migrating proteins is 13 kDa.

Complementation of the Fur$^-$ phenotype in the manganese-resistant PA103 mutants. To examine whether *P. aeruginosa fur* could complement the mutant phenotype of PA103M, plasmid pKTFUR was mated into PA103M. PA103M containing multiple copies of pKTFUR no longer produces constitutive levels of pyoverdin compared with PA103M containing a vector control (pKT230) (Table 3). In addition, multiple copies of pEHFUR are able to restore normal siderophore production to strain PA103M (data not shown).

The ability of pEHFUR to restore normal regulation of exotoxin A production in strain PA103M was also investigated by Western blot analysis (Fig. 5C). Normal regulation of exotoxin A by iron is restored in PA103M containing multiple copies of pEHFUR in comparison with PA103M that contains a vector control (Fig. 5C). Furthermore, β-galactosidase activities in PA103M carrying a *toxA::lacZ* fusion integrated into its chromosome at the *toxA* locus (PA103MC) indicate that PA103MC containing a vector control has virtually no iron regulation of β-galactosidase production as compared with PA103MC that contains pEHFUR. PA103MC(pEHFUR) has normal iron regulation at the later time points (Fig. 5D). Curiously, when plasmid pKTFUR was tested for its ability to restore normal iron regulation of exotoxin A in strain PA103M, it did not have an effect on the constitutive production of exotoxin A by that strain (data not shown). It should also be noted that when PA103M contains multiple copies of pEHFUR or pKTFUR, it becomes susceptible to manganese, like wild-type PA103.

DISCUSSION

Genes that are homologous to *E. coli fur* recently have been cloned from *V. cholerae* and *Y. pestis* (31, 45, 46). In this paper, we report the cloning of a gene from *P. aeruginosa* that is both structurally and functionally similar to *E. coli fur*. *P. aeruginosa fur* has some very distinct features, particularly at the C terminus. *E. coli*, *V. cholerae*, and *Y. pestis* Fur proteins have at their C termini amino acid

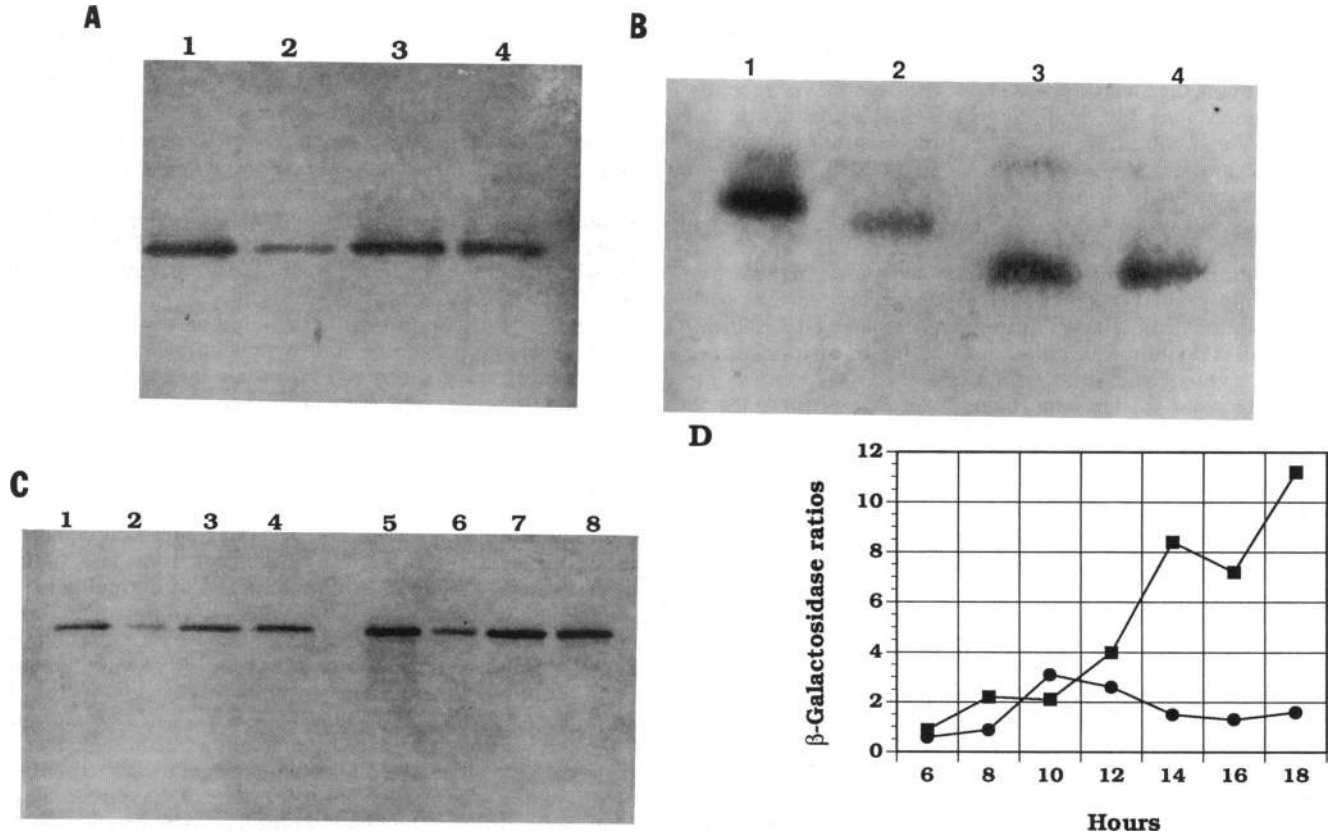

FIG. 5. (A) Western blot analysis of exotoxin A production by *P. aeruginosa* PA103 (lanes 1 and 2) and PA103M (lanes 3 and 4). Cultures were grown for 13 h, and culture supernatants were analyzed by Western blotting by probing with an affinity-purified exotoxin A antiserum. Odd-numbered lanes represent cultures that were grown in iron-deficient medium (DTSB), and even-numbered lanes represent cultures that were grown in iron-rich medium (DTSB plus 36 μM iron). (B) Western blot analysis of Fur from *P. aeruginosa* PA103 (lane 1) and three independently isolated fluorescent, manganese-resistant PA103 mutants (lanes 2 through 4). PA103M is represented in lane 2. Whole-cell extracts were made from an equal number of cells from cultures that were grown for 16 h. Extracts were probed with an anti-*E. coli* Fur serum. There were no differences in the migration of other proteins produced by any of these strains. (C) Western blot analysis of exotoxin A production by *P. aeruginosa* PA103M(pEHFUR) (lanes 1, 2, 5, and 6) and PA103M(pUCRSF) (lanes 3, 4, 7, and 8). Cultures were grown for 16 h (lanes 1 through 4) or 18 h (lanes 5 through 8), and culture supernatants were analyzed for exotoxin A production by Western blotting with an affinity-purified exotoxin A antiserum. Odd-numbered lanes represent cultures that were grown in low-iron medium and even-numbered lanes represent cultures that were grown in high-iron medium. (D) Ratios of β-galactosidase activities at the indicated times for cultures of PA103MC(pEHFUR) (■) and PA103MC(pUCRSF) (●) grown in high- and low-iron media. PA103MC contains a *toxA*::*lacZ* fusion integrated into its chromosome at the *toxA* locus and was made as previously described (52).

sequences that have been hypothesized to function in metal binding or DNA binding (26, 28). While this sequence is absent from *P. aeruginosa Fur*, the C terminus does have a highly basic sequence that could be involved in DNA binding (Fig. 3).

We were unable to make a *fur* null mutation in *P. aeruginosa* by standard genetic techniques. This is in contrast to *E. coli*, for which *fur* null mutants have been described (7, 13). It is possible that a complete loss of a functional *fur* gene is lethal in *P. aeruginosa*. Why this would be the case is not clear, but it could be due to the more aerobic nature of *P. aeruginosa* as compared with *E. coli* and the preferred use of succinate as a carbon source by *P. aeruginosa*. *P. aeruginosa* Fur mutants have been made in this study by manganese selection. These mutants grow more slowly than wild-type PA103, suggesting that this type of mutation in *P. aeruginosa* is in some way affecting functions vital for cell growth. The Fur mutants analyzed in this study all produce Fur proteins that migrate faster than wild-type Fur proteins in Western blots (Fig. 5B). It is possible that the altered Fur proteins maintain partial function in the regulation of a critical process necessary for the survival of *P. aeruginosa*. In this regard, it is interesting that while the mutation in PA103M affects exotoxin A regulation and siderophore regulation, the mutations in the other PA103 manganese-resistant mutants shown in Fig. 5B (lanes 3 and 4) affect only siderophore regulation and not exotoxin A regulation (50b).

It is not clear why resistance to manganese appears to coincide with mutations in *fur*. Manganese is mutagenic and affects the fidelity of DNA polymerase (5, 15, 29, 39). It is possible the *fur* gene is highly sensitive to the mutagenic action of manganese. Another possibility is that mutations in *fur* somehow enable bacteria to survive otherwise lethal concentrations of manganese and that these *fur* mutants are selected for by plating on high concentrations of manganese. All three of the three fluorescent manganese-resistant strains analyzed in this study produce altered Fur proteins. These mutant Fur proteins could be the result of premature stop codons being introduced into the *fur* gene, different start

sites being recognized in the *fur* gene, or a posttranscriptional processing event that results in a protein that migrates faster than the wild type. However, we have recently determined that the mutation in PA103M results in an Ala-to-Val substitution in Fur near the His-rich region and that the mutation in both of the other manganese-resistant mutants results in an Asp-to-Gly substitution in the coding sequence of Fur (50a).

We previously found that the *toxA* gene of *P. aeruginosa* PA103 could be regulated by multiple copies of *E. coli fur* (38). This regulation appeared to occur by repression of transcription from the P1 promoter of the *regAB* operon, although it could not be ruled out that it was also occurring at the level of the *toxA* promoter. If *P. aeruginosa fur* regulates *toxA* in the same manner as *E. coli fur* does, it would be expected that transcription from the P1 promoter of the *regAB* operon would be constitutive in the manganese-resistant Fur mutants. It is interesting that there is no sequence in the promoter region of *regAB* that resembles the consensus Fur-binding site. This may indicate that *P. aeruginosa* Fur recognizes a sequence different from that recognized by *E. coli* Fur or that it may regulate another gene involved in the regulation of transcription from the *regAB* P1 promoter. Because *P. aeruginosa fur* can complement an *E. coli fur* mutant, it does not seem likely that it recognizes a DNA-binding site that would be dramatically different from the consensus Fur-binding site. However, since *P. aeruginosa* has a higher G+C content than *E. coli*, it is possible that *P. aeruginosa* Fur may recognize a binding site that is substantially different from the *E. coli* Fur-binding site. There is a site in the *toxA* gene that resembles a Fur-binding site, and this might be a site that is recognized by *P. aeruginosa* Fur (38). This site, however, lacks the dyad symmetry normally found in *E. coli* Fur-binding sites (3, 7, 12, 14). The ability to bind to a site that is not like the consensus Fur-binding site could be related to the distinct C terminus of *P. aeruginosa* Fur.

Another difference between *E. coli* Fur and *P. aeruginosa* Fur is in their abilities to regulate exotoxin A production in strain PA103C under low-iron conditions. Multiple copies of *E. coli fur* in PA103C inhibited exotoxin A production even in low-iron medium (38). This is in contrast to *P. aeruginosa fur*, which does not affect exotoxin A production in low-iron medium when present in multiple copies in strain PA103C (Fig. 4). One possible explanation for these differences is that *P. aeruginosa* Fur may have no or very low affinity for its target promoters in low-iron medium, whereas *E. coli* Fur may have some affinity for its target promoters in low-iron medium. It has been reported that *E. coli* Fur does have some sequence-specific binding when present in high concentrations even without iron (14). Also, as shown in Table 2, *P. aeruginosa fur* does not repress β-galactosidase activity as much as *E. coli fur* does in low-iron medium when present in multiple copies in *E. coli* 1618. This may indicate that *P. aeruginosa* Fur has a lower affinity for its target promoters under low-iron conditions than *E. coli* Fur does. Another possibility is that *P. aeruginosa* Fur is somehow regulated in *P. aeruginosa* in a manner that makes it unable to function under low-iron conditions and that *E. coli* Fur is not subject to this control. It is interesting that the Fur-like gene *dtxR* from *Corynebacterium diphtheriae*, which regulates diphtheria toxin production, also has no effect on diphtheria toxin production or siderophore production in low-iron medium when present in multiple copies in a wild-type strain of *C. diphtheriae* (42).

One curious finding was that a multicopy plasmid (pKTFUR) carrying *P. aeruginosa fur* on a large DNA fragment (3.1 kb) was able to complement only siderophore production in strain PA103M and not exotoxin A production. When a smaller DNA fragment (1.8 kb) containing *P. aeruginosa fur* was used, it was able to complement both siderophore and exotoxin A production in strain PA103M. It is possible that there are other genes present on pKTFUR which somehow interfere with the ability of *P. aeruginosa fur* to repress exotoxin A production in strain PA103M. It is also possible that the expression of *P. aeruginosa fur* from pKTFUR is less than that from pEHFUR and that *P. aeruginosa* Fur has a greater affinity for the operator sequences of the pyoverdin and pyochelin genes. It may be that higher concentrations of Fur are needed to repress exotoxin A production than are needed to repress siderophore production. Schmitt and Holmes (42) also found that a large DNA fragment containing the *dtxR* gene complemented siderophore production in a *dtxR* mutant strain of *C. diphtheriae* better than it complemented diphtheria toxin production. When a smaller DNA fragment was used by these investigators, regulation of diphteria toxin production in the mutant strain was almost returned to normal (42).

The ability of *P. aeruginosa fur* to complement both siderophore production and exotoxin A production in PA103M indicates that *P. aeruginosa fur* is a global regulator in *P. aeruginosa*, as *fur* is in other genera of bacteria (18, 21, 26, 47). The regulation of *regAB* and *toxA* by *P. aeruginosa fur* could be complex, since there is not a Fur-binding site in the *regAB* P1 promoter. This is in contrast to the regulation of diphtheria toxin production by *dtxR* in *C. diphtheriae*. The DtxR protein inhibits diphtheria toxin production in high-iron medium directly, by binding to a Fur-like consensus sequence in the promoter region of the *tox* gene, which encodes diphtheria toxin (50).

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