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Gene repression by the ferric uptake regulator in *Pseudomonas aeruginosa*: Cycle selection of iron-regulated genes

(DNA–protein interaction/Fur-box/systematic evolution of ligands by exponential enrichment/siderophore receptors/σ factors)

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ABSTRACT **The expression of at least 24 distinct genes of *Pseudomonas aeruginosa* PAO1 is under direct control of the "ferric uptake regulator" (Fur). Novel targets of the Fur protein were isolated in a powerful SELEX (systematic evolution of ligands by exponential enrichment)-like cycle selection consisting of *in vitro* DNA–Fur interaction, binding to anti-Fur antibody, purification on protein G, and PCR amplification. DNA fragments obtained after at least three exponential enrichment cycles were cloned and subjected to DNA mobility-shift assays and DNase I footprint analyses to verify the specific interaction with the Fur protein *in vitro*. Iron-dependent expression of the corresponding genes *in vivo* was monitored by RNase protection analysis. In total, 20 different DNA fragments were identified which represent actual *Pseudomonas* iron-regulated genes (PIGs). While four PIGs are identical to already known genes (*pfeR*, *pvdS*, *tonB*, and *fumC*, respectively), 16 PIGs represent previously unknown genes. Homology studies of the putative proteins encoded by the PIGs allowed us to speculate about their possible function. Two PIG products were highly similar to siderophore receptors from various species, and three PIG products were significantly homologous to alternative σ factors. Furthermore, homologs of the *Escherichia coli* ORF1-*tolQ*, *nuoA*, stringent starvation protein Ssp, and of a two-component regulatory system similar to the *Pseudomonas syringae* LemA sensor kinase were identified. The putative gene products of seven additional PIGs did not show significant homologies to any known proteins. The PIGs were mapped on the *P. aeruginosa* chromosome. Their possible role in iron metabolism and virulence of *P. aeruginosa* is discussed.**

The opportunistic pathogen *Pseudomonas aeruginosa* is highly capable of regulating virulence-associated genes in response to specific environmental conditions, such as limitation of iron in a host, where this micronutrient is bound to transferrin and lactoferrin (for review see ref. 1). *P. aeruginosa* has a relatively high demand for iron, since it prefers an aerobic metabolism requiring respiratory enzymes that need iron for their function. Therefore, *P. aeruginosa* has evolved powerful mechanisms to acquire iron, including (*i*) the release of two types of siderophores, pyochelin (2) and pyoverdin (3), which scavenge the low amount of free Fe(III) and are taken up by specific receptors (4, 5); (*ii*) the secretion of cytotoxic exotoxin A, which can lead to the release of iron from damaged host cells (6, 7); and (*iii*) the production of extracellular proteases which degrade the iron-binding proteins of the host (8). In *P. aeruginosa*, as in numerous other pathogenic organisms, the expression of genes involved in iron acquisition is strictly regulated in response to the iron concentration in the microenvironment (1, 9, 10). The central role in the control of iron-regulated genes has been assigned to the "ferric uptake regulator" (Fur) protein, which acts as an iron-responsive, DNA-binding repressor protein (for reviews, see refs. 11 and 12). Employing Fe(II) as a cofactor, the Fur protein binds to the palindromic consensus sequence GATAATGATAATCATTATC, the so-called "Fur-box", which has first been identified in the promoter regions of iron-regulated genes of *Escherichia coli* (13). Binding of Fur–Fe(II) to operator sequences results in repression of these genes, while under low iron conditions, Fur is released and transcription takes place. Well-conserved homologs of Fur have been found in a large number of Gram-negative bacteria, including several important human pathogens (for review see ref. 14). We previously reported on the identification of a Fur homolog in *P. aeruginosa* (15), on the cloning and sequencing of the *fur* gene (16), and on the purification and activity of the Fur repressor (14). Fur appears to be essential in *P. aeruginosa*, however, the phenotypes of strains expressing mutant Fur proteins have been characterized (17). In this report, we demonstrate that Fur regulates at least 24 genes in *P. aeruginosa*, some of which may play an important role during pathogenesis. The novel Fur-regulated genes were identified in a powerful *in vitro* cycle selection procedure that may prove helpful in similar situations where targets for a given regulatory factor have to be found.

MATERIALS AND METHODS

DNA Fragment Library. Chromosomal DNA of *P. aeruginosa* was partially digested with *Hpa* II, and DNA fragments in the size range of 200–500 bp were recovered from a preparative 1.2% agarose gel. Complementary linkers (5′-CGGAGTGACTCTTGACCTCGACTAGTGCA and 5′-TGCACTAGTCGAGGTCAAGAGTCACTC) containing a *Hpa* II protruding end (CG) and an internal *Spe* I restriction site (ACTAGT) were prepared on a Oligo1000 DNA synthesizer (Beckman). After annealing and phosphorylation, 200 pmol (4 μg) of the linkers were ligated to 4 pmol (1 μg) of *Hpa* II-digested DNA (18). After treatment with *Spe* I to eliminate linker multimers, the DNA fragments were separated from the nonincorporated linkers in a preparative agarose gel.

Cycle Selection Procedure. The DNA fragment library was amplified and radiolabeled by PCR in a Perkin–ElmerCetus thermal cycler, with 25 cycles of denaturing (1 min at 94°C), annealing (30 sec at 56°C), and extending (40 sec at 72°C). The PCR mixture (50 μl) contained 60 mM Tris·HCl (pH 8.5), 15 mM $(NH_4)_2SO_4$, 3.5 mM $MgCl_2$, 1–10 ng of template DNA, 25 pmol of the linker-specific primer 5′-TCGAGGTCAAGAGTCACTCC, 0.15 mM dNTPs, 1 μl of [α-^{32}P]dCTP, and 2 units of *Taq* DNA polymerase (GIBCO/BRL). The amplified DNA fragments were purified in a preparative 1.2% agarose gel. To form DNA–Fur complexes, 2 μg of DNA were mixed into 500 μl of binding buffer (10 mM Bis-Tris borate, pH 7.5/40 mM KCl/0.1 mM $MnSO_4$/1 mM $MgSO_4$/50 μg of bovine serum albumin per ml/10 μg of poly(dI·dC) per ml/10% glycerol),

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Abbreviations: PIG, *Pseudomonas* iron-regulated gene; Fur, ferric uptake regulator; ORF, open reading frame.

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and purified Fur protein was added to a final concentration of 200 nM. After incubation of the binding reaction for 30 min at 37°C, rabbit anti-Fur serum (30 μl) was added, and the incubation was continued for 30 min at 37°C. The mixture was loaded on a 1-ml HiTrap Protein G column (Pharmacia) equilibrated with binding buffer. The column was washed with 3 ml of binding buffer. Bound DNA–Fur–anti-Fur complexes were eluted with 1.5 ml of 0.1 M glycine·HCl, pH 2.7. Fractions of 0.25 ml were collected and immediately neutralized by the addition of 25 μl of 1 M Tris·HCl, pH 9. The DNA-containing fractions, as monitored by scintillation counting, were combined. Release of the DNA fragments from Fur was achieved by heating 2–10 μl of the DNA at 94°C for 2 min prior to PCR to generate the DNA fragments for the next cycle.

DNA and RNA Manipulations. The DNA fragments obtained after three to five cycles of selection for Fur binding were cloned into pCRII (Invitrogen). Individual clones were excised from pCRII with either a combination of *Xho* I/*Sst* I or *Hin*dIII/*Eco*RV and selectively labeled at the *Xho* I or the *Hin*dIII site by the Klenow fill-in reaction using radiolabeled [α-^{32}P]dCTP. After gel purification, single labeled fragments were subjected to mobility shift and DNase I footprint assays using purified Fur (14). Actual Fur targets were sequenced by the dideoxy chain termination method (19) using Sequenase (United States Biochemical) and M13 primers. Radiolabeled DNA probes were prepared from purified DNA fragments obtained in the cycle selection by the random primer method (20) using the RadPrime DNA labeling system (GIBCO/BRL). These probes were used for the physical mapping of the corresponding genes on the *P. aeruginosa* chromosome (21–23) and for colony hybridization (24). Chromosomal DNA fragments containing the corresponding genes were isolated from a *P. aeruginosa* PAO1 plasmid gene library that had been constructed by ligating 3–9 kb DNA fragments obtained by partial digestion of chromosomal DNA with *Taq* I into the *Cla* I site of pBluescript SK+. DNA sequence extending upstream and downstream of the original cycle-selected DNA fragments was determined from these chromosomal clones by using outreading oligonucleotide sequencing primers. RNA probes were generated *in vitro* from the original cycle-selected DNA fragments or from suitable PCR fragments that covered the Fur-box plus at least 120 bp adjacent to the Fur box, using the Riboprobe system (Promega). Separate radiolabeled runoff transcripts from the SP6 and T7 promoters that flank the polylinker of pCRII were used for RNase protection analyses to detect putative transcripts starting close to the Fur box.

RESULTS

Cycle Selection of DNA Fragments Bound by the Fur Protein. DNA fragments harboring Fur-box-like elements were isolated from a large pool of 0.2- to 0.5-kb genomic fragments following the powerful cycle selection depicted in Fig. 1. In this procedure, DNA fragments with affinity to purified Fur are exponentially enriched in consecutive cycles of DNA–Fur interaction, binding to antibody, purification of the tripartite complexes on a protein G column, and PCR amplification of the enriched DNA pool. After three to five selection cycles, the remaining DNA fragments were cloned into pCRII. Individual DNA fragments were subjected to mobility-shift assays, resulting in the identification of 30 different fragments that were bound *in vitro* by purified Fur at a concentration of 100 nM or less.

In a model system using a mixture of a Fur-box-containing fragment and a 20-fold excess of an irrelevant DNA fragment, the conditions for the binding of the DNA to Fur, the binding to anti-Fur antibody, and the affinity purification on protein G Sepharose were optimized to obtain a ≥5-fold enrichment of the Fur box-containing fragment per cycle (data not shown). Since the enrichment is exponential, five cycles would result in a roughly 3000-fold enrichment. With the complex pool of 0.2- to 0.5-kb genomic *P. aeruginosa* DNA fragments as the starting material, the enrichment for Fur targets was high in the first two cycles and decreased in later cycles. After a single cycle of selection, 5% of the resulting DNA fragments were already bound by Fur, and after two and three consecutive cycles, this increased to 25% and 50%, respectively.

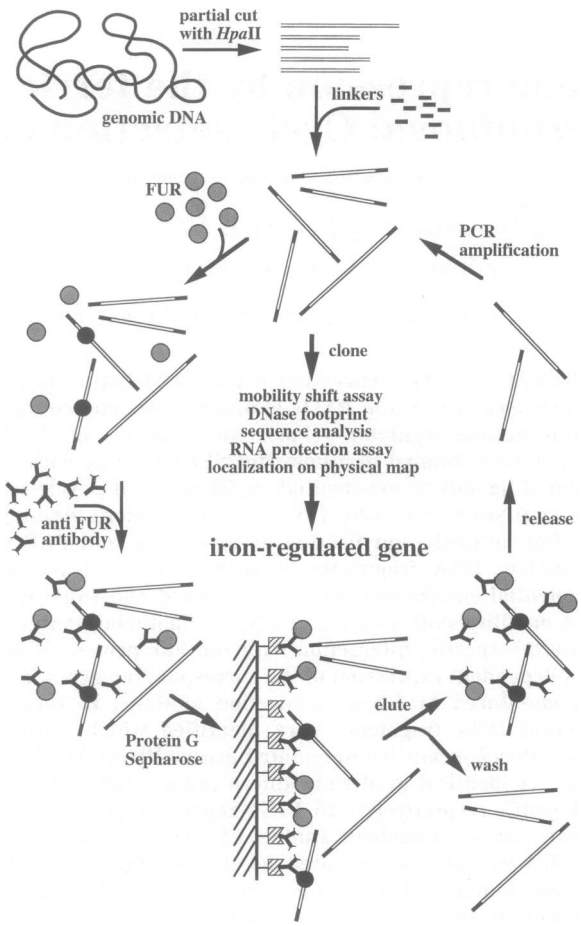

FIG. 1. Cycle selection procedure. Putative DNA targets for the Fur protein are exponentially enriched in consecutive cycles of DNA–Fur interaction, binding to anti-Fur antibody, purification of the complexes on protein G Sepharose, and PCR amplification. The DNA fragments for the starting pool were obtained by partial digestion of chromosomal *P. aeruginosa* DNA with *Hpa* II and contain linkers at both ends (shown as black tails).

Specificity of the Fur–DNA Interaction. DNA sequence analysis of the 30 clones that were gel-shifted by purified Fur indicated that all of them contained a Fur-box of 53–79% identity, which is a match of 10/19 to 15/19, to the *E. coli* Fur-box consensus sequence GATAATGATAATCATTATC. Some fragments contained two overlapping or two tandemly organized Fur-boxes. DNase I footprint analyses were carried out to verify the specificity of the Fur–DNA interaction. Of the 30 cycle-selected DNA fragments that were gel-shifted, only 18 resulted in a DNase I footprint. The area protected from cleavage by DNase I covered the 19-mer Fur-box sequence plus a variable number, typically 5–10 bp, on either side of the Fur-box. A compilation of the Fur-boxes contained within those fragments that resulted in a specific DNase I footprint gave evidence that the Fur-box consensus sequence of *P. aeruginosa* is identical to the one of *E. coli* (Fig. 2).

Those DNA fragments that were gel-shifted, but did not result in a DNase I footprint, were generally very A+T-rich, typically containing 60% of A+T over a stretch of 400 bp. Considering that the Fur-box is very A+T-rich itself (79% A+T), these A+T-rich fragments were obviously bound by Fur rather nonspecifically due to the presence of incidental Fur-box-like elements.

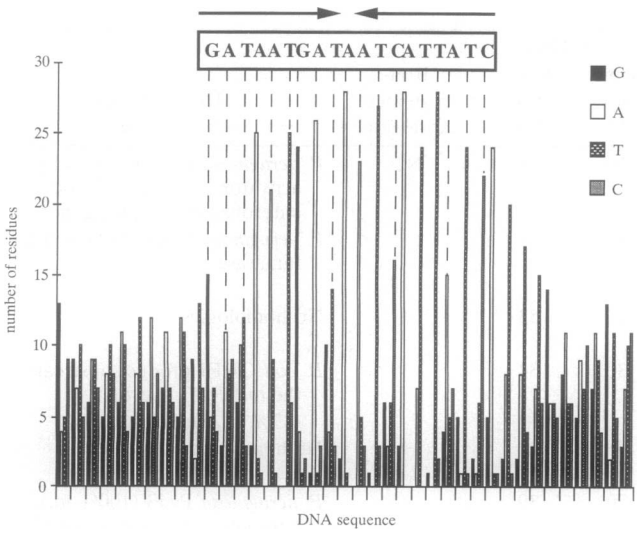

FIG. 2. Compilation of Fur-box elements of *P. aeruginosa*. A total of 31 Fur-box-containing DNA sequences from newly identified Fur targets and from additional known iron-regulated genes were aligned. Where tandemly organized or overlapping Fur-boxes were detected, both were used for the alignment. The occurrences of A, T, C, and G in each position within and adjacent to the Fur-box were counted and are presented as bars. The 19-mer consensus sequence GATAATGATAATCATTATC is shown in the box, and its palindromic organization is indicated by arrows.

Iron Regulation *in Vivo*. The Fur-boxes of the cycle-selected DNA fragments were generally localized within an A+T-rich region representing a typical promoter and were flanked by DNA sequence of high G+C content, indicating possible coding sequence; however, the direction of putative Fur-regulated genes remained unclear. Therefore, additional DNA sequence was determined on both sides of the Fur-box by using larger genomic DNA clones isolated from a *P. aeruginosa* plasmid gene library. Putative open reading frames (ORFs) were identified according to the *P. aeruginosa* codon preference (25). The expression of these ORFs in response to iron starvation was analyzed by RNase protection comparing RNA from cells grown on high versus low iron conditions. ORFs that were expressed in an iron-dependent manner were designated *Pseudomonas* iron-regulated genes (PIGs). The properties of the DNA sequences representing actual PIGs are summarized in Table 1. The PIGs were found to be widely scattered on the physical map of the *P. aeruginosa* PAO1 chromosome. All cycle-selected DNA fragments that resulted in a DNase I footprint appeared to represent genes that were expressed in response to iron limitation *in vivo*, as judged by RNase protection. The Fur-mediated iron regulation of two representative genes, *pig31* and *pig12*, is demonstrated by mobility-shift assays, DNase I footprint analyses, and RNase protection analyses in Fig. 3. Cycle-selected DNA fragments that resulted in a gel-shift, but not in a footprint, did not exhibit iron-regulated expression *in vivo* (data not shown). In the *P. aeruginosa* PAO-C6 mutant strain producing an altered Fur protein (17), expression of *pig12* and *pig19* was not repressed under high iron conditions (data not shown), giving direct evidence that iron regulation is indeed mediated by the Fur repressor at least in the case of these two PIGs.

Sequence Homologies of the PIG Products to Bacterial Proteins. The PIGs that are clearly Fur regulated, as judged from DNase footprint analyses and RNase protection, fell into three groups. The first group includes four already known *P. aeruginosa* genes, namely *pfeR* (*pig2*), *fumC* (*pig3*), *pvdS* (*pig8*), and *tonB* (*pig13*), which prove the value of the applied cycle selection method. PfeR acts together with PfeS as a two-component regulatory system involved in the transcriptional control of the *pfeA* enterobactin receptor gene (26). The *fumC* gene encodes a recently identified iron-regulated fumarase and is located in an operon together with *sodA* encoding a superoxide dismutase (D. J. Hassett, personal communication). PvdS is an alternative σ factor required for the production of the siderophore pyoverdine and its receptor (27). TonB is a key component in the transport of ferric siderophore complexes through the cytoplasmic membrane, and the *tonB* gene of *P. aeruginosa* has recently been identified (K. Poole, S. Neshat, D. E. Heinrichs, and C. R. Dean, personal communication). The second group of PIGs represents nine novel *P. aeruginosa* genes encoding putative proteins with significant homologies to known bacterial proteins, allowing to speculate about their possible functions (Table 1). The protein encoded by the complete ORF within *pig6* has 37% overall identity to the product of ORF1 upstream of the *tolQRAB* operon of *E. coli*, which is involved in the transport of colicins and phage DNA into the cell (28). *pig12*–ORF2 encodes a protein without homology to any known bacterial protein; however, part of an upstream ORF (*pig12*–ORF1) was detected, which encodes a putative protein with 27% identity to the *Yersinia enterocolitica* FoxA ferrioxamine receptor (29), 26% identity to the *E. coli* ferric enterobactin receptor FepA (30), and 28% identity to the *E. coli* FhuA ferrichrome receptor (31). The *pig12*–ORF1, although not directly affected by the Fur-box present in the *pig12* region, was also found to be expressed in response to iron limitation when a riboprobe covering the start of *pig12*–ORF2 and the end of the upstream *pig12*–ORF1 was used (data not shown). The *pig31*-encoded polypeptide contains an N-terminal signal sequence typical for exported proteins (32), and the first 111 aa from Pig31 exhibit 25% identity to both the *Erwinia chrysanthemi* Fct ferrichrysobactin receptor (C. Sauvage, T. Franza, and D. Expert, GenBank accession no. X87967) and the *E. coli* FhuE receptor (33). Upstream of *pig31*, a complete ORF was found to encode a protein of 66% identity to the *E. coli* CyoE cytochrome *o* ubiquinol oxidase subunit (34); however, the expression of this *cyoE* homolog was not iron-regulated. The gene product of *pig14* is 55% identical to the NuoA subunit of the proton-translocating NADH-ubiquinone oxidoreductase of *E. coli* (35). The gene products of *pig17*, *pig25*, and *pig32* exhibit a significant degree of homology to alternative σ factors of the ECF (extracytoplasmic factor) subfamily (36). An alignment of the proteins encoded by *pig17*, *pig25*, and *pig32* with the characteristic regions within alternative σ factors is presented in Fig. 4. Pig17, Pig25, and Pig32 have overall sequence identities of 42, 22, and 31%, respectively, to the FecI protein of *E. coli*, which was initially identified as a regulator of the citrate-dependent Fe(III) transport system and acts together with FecR in a two-component regulatory system (37). Downstream of *pig17*, a transcriptionally coupled ORF was identified encoding a 36-kDa protein with 29% sequence identity to the *E. coli* FecR sensor protein (data not shown). The protein encoded by *pig25* is also 17% identical to *M. xanthus* CarQ, a regulator of carotenoid biosynthesis (41). The Fur-box within the *pig17* region also affected the transcription of the divergent *pig17*–ORF2, which encodes a protein similar to the stringent starvation protein (SSP) of *E. coli* (42). The Fur-box within *pig19* is located between two divergent genes. *Pig19*–ORF2 encodes a sensor-kinase of 52% identity to the *Pseudomonas syringae* LemA sensor-kinase involved in plant disease (43, 44); however, its expression was not iron-dependent. *pig19*–ORF1 encodes an OmpR-like response regulator which is 61% identical to the PfeR regulator of the ferrienterobactin uptake system in *P. aeruginosa* (26), and the expression of *pig19*–ORF1 was strictly Fur controlled. Both proteins encoded by the divergent ORFs in the *pig19* region harbor the conserved residues typical for histidine kinases and for phosphorylation-activated response regulators, respectively (45). The third group includes seven novel PIGs (*pig4*, *pig7*, *pig9*, *pig12*–ORF2,

Table 1. Iron-regulated genes of *P. aeruginosa*

Gene	Map location*	Fur-box element†	Match out of 19-mer	Palindrome length‡	Gel shift with Fur,§ nM	DNase I footprint, bp	Iron-regulated expression¶	Homology to other proteins**
pig2	S-P, D-D 42.0–42.8′	acgAAcGtgAATCATTcTC	12	3	10	31	ND	*P. aeruginosa* PfeR (100% id.), activator of the *pfeA* ferric enterobactin receptor gene
pig3	S-E, D-I 71.3–75.0′	GATAATGAgAtTgATTATt GAaAAcaATAATCAaTcTC	15 14	6 5	10	37	+	*P. aeruginosa* FumC (100% id.), fumarase
pig4	S-F, D-A 66.1–70.2′	GAcAtTGAgAtTCAaTgaC ataAtTGAgAATCgTTATt	12 12	7 4	10	53	+	No homologies
pig6	S-F, D-A 66.1–70.2′	GAagATGgTAATtAaTtgC	12	4	30	34	+	*E. coli* ORF1 product upstream of the *tolQRAB* operon (37% id. overall)
pig7	S-E, D-I 71.3–75.0′	tccAATGcaAATCAaTATC GccAATGATAtTgATTtgC	13 13	3 5	30	32	+	No homologies
pig8	S-J, D-B 45.9–48.8′	GtaAtTGAcAATCATTATC	15	5	20	39	+	*P. aeruginosa* PvdS (100% id.), alternative σ factor required for pyoverdine synthesis
pig9	ND	GATAATGAgAATagTTATt tgTAATaATAActATTcTC	15 13	5 5	10	27	ND	No homologies
pig12	S-E, D-I 71.3–75.0′	ttTAtcGcaAcTgATTATC	11	2	10	29	+(ORF1) +(ORF2)	ORF1 product: Siderophore receptors (26–28% id. over 88–100 aa); ORF2 product: no homologies
pig13	S-K, D-F_2 10.5–12.8′	ctgAATGATAATaATTATC tcTtcTGATAATtATTATC	15 14	5 4	10	29	ND	*P. aeruginosa* TonB (100% id.)
pig14	S-N, D-C 59.2–61.3′	GcaAAcGATAtTCATcATC	14	4	30	33	Weak	*E. coli* NuoA subunit of NADH-ubiquinone oxidoreductase (55% id. over 120 aa)
pig17	S-H, D-F_1 19.1–20.2′	aAaAAcGAgAATtATTcgC	12	3	30	32	+(ORF1) +(ORF2)	Alternative σ factors (42% id. overall to FecI) Divergent ORF2: stringent starvation protein SSP
pig18	S-X, D-B 48.8–49.6′	GATAtTaATAccCATTtca	12	2	100	33	Transcripts undetectable	No homologies
pig19	S-D, D-B 49.6–52.3′	acgAtTGcTAATCAaTcTt	11	7	30	34	+(ORF1) −(ORF2)	*P. aeruginosa* PfeR regulator (61% id. over 132 aa) Divergent ORF2: LemA (52% id. over 123 aa)
pig20	S-B, D-J/K 0.9–6.6′	GgaAATGAgAATCATTATt	15	5	30	30	+	No homologies
pig23	S-E, D-E 0.0–0.3′	ctgAATGAaAccCAaTcTt	10	4	100	29	+	No homologies
pig25	S-C, D-M 31.5–33.3′	acgAtTGAaAATCATTATC ttTtATGATAATgATTtTC	14 14	4 4	30	35	+	Alternative σ factors (22% id. overall to FecI, 17% id. overall to CarQ)
pig31	S-M, D-A/C 61.3–63.8′	GtTAtTGAgAATCATTggC	14	5	30	35	+	Ferric siderophore receptors (25% id. over 111 aa to Fct and FhuE)
pig32	S-J, D-B 45.9–48.8′	ctTgATGAgAATtATTATg tAaAtTcATAATaATTcTC	13 13	5 5	100	38	+	Alternative σ factors (31% id. overall to FecI) Divergent ORF: no homologies
fpvA	S-J, D-B 45.9–48.8′	GcTgATcAcgATgATggTC GATcAcGATgATggTctTg	11 11	5 5	ND	ND	ND	*P. aeruginosa* FpvA ferripyoverdine receptor
pfeA	S-P, D-D 42.0–42.8′	tcaAATaAcAATCAaTATC	13	3	ND	ND	ND	*P. aeruginosa* PfeA ferric enterobactin receptor
fptA	S-A, D-F 21.7–24.1′	cATAATGATAAgCATTATC	17	7	ND	ND	ND	*P. aeruginosa* FptA ferricpyochelin receptor
pchR	S-A, D-F 21.7–24.1′	GgaAATGAgAtTtATTATC GAgAtTtATtATCATTggC	14 13	5 5	15	33	ND	*P. aeruginosa* PchR regulator of pyochelin and pyochelin receptor synthesis
Consensus		GATAATGATAATCATTATC	19	9				

The DNA fragments harboring a Fur-box were isolated by cycle selection. The PIGs were localized on the physical map, and their Fur-box-like elements were compared with the consensus Fur-box sequence and analyzed for the palindrome length. The concentration of purified Fur resulting in a mobility shift of the fragments in gel retardation assays, the area of protection in DNase I footprint analyses, and the iron-regulated expression are indicated. The corresponding proteins encoded by the PIGs were analyzed for homologies to known bacterial proteins. Included in the list are four iron-regulated *P. aeruginosa* genes (*fpvA*, *pfeA*, *fptA*, and *pchR*) harboring a Fur-box which were not among the PIGs isolated in this study. ND, not determined.

(Table footnotes appear on the opposite page.)

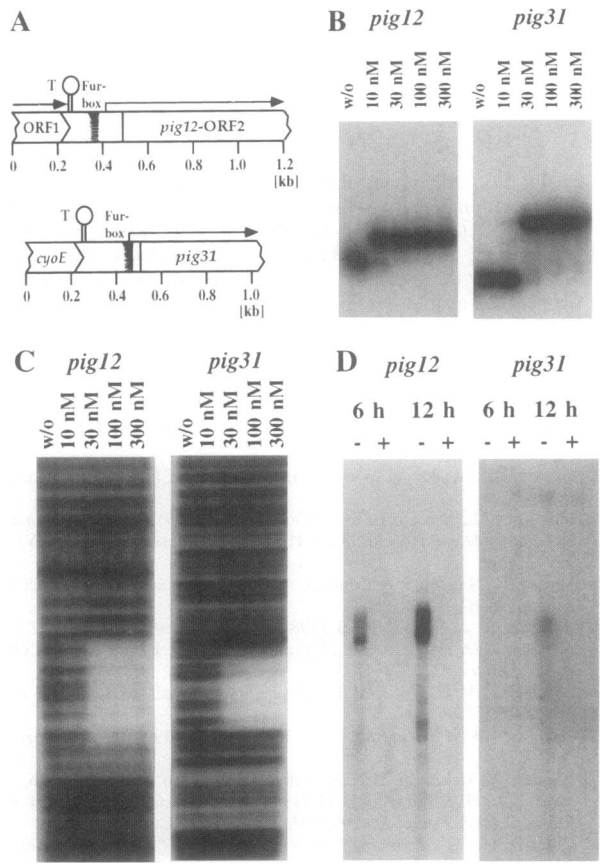

FIG. 3. Fur regulation of *pig12* and *pig31*. The genetic organization of the *pig12* and *pig31* regions is depicted in *A*. Direct binding of Fur to the corresponding promoter regions is demonstrated in mobility-shift assays (*B*) and DNase I footprint analyses (*C*). The iron-dependent expression of *pig12* and *pig31 in vivo* is shown by RNase protection analyses (*D*) using equal amounts of RNA from cells grown for 6 h and 12 h under low (−) or high (+) iron conditions.

pig18, *pig20*, and *pig23*) without homology to any known genes. Four additional iron-regulated genes of *P. aeruginosa* that were not isolated by the cycle selection procedure are included in Table 1, namely *fpvA* (5), *pfeA* (46), *fptA* (4), and *pchR* (47). Careful bookkeeping reveals that there are at least 24 Fur-regulated genes in *P. aeruginosa* by now.

DISCUSSION

In this study, we present a powerful cycle selection strategy leading to the isolation of 16 novel genes controlled by the Fur repressor. The biochemical *in vitro* method makes use of the specific interaction of the Fur protein to DNA and could be a general strategy to find the targets for any given regulatory protein. The procedure is derived from the idea of systematic evolution of ligands by exponential enrichment (SELEX), which is a method to isolate RNA species exhibiting specific binding properties to a given target (e.g., protein or low molecular weight compound) from a highly complex pool of synthetic RNA molecules (48). The prerequisites for SELEX and for SELEX-like procedures are a representative starting pool, a quick binding assay, and an amplification scheme (e.g., PCR) of the enriched pools between the cycles. Other strategies to isolate targets of a given regulatory protein have been described. The screening of *TnphoA* or *lacZ* operon fusions has been used most frequently, and a further development of this *in vivo* method is the titration assay that is based on multiple plasmid-borne copies of a consensus binding site deregulating the genomic target genes by titrating the responsible regulatory protein. The Fur titration assay allowed the isolation of many targets of the Fur repressor in *E. coli* (49) and in *Salmonella typhimurium* (50). An alternative *in vitro* method is based on the migration properties of DNA fragments in a two-dimensional gel where the first dimension is performed in the presence of the DNA-binding protein and the second dimension is carried out at high temperature to relieve DNA–protein interaction, thus allowing to detect and purify target DNA fragments migrating off the diagonal in such a gel (51). The conditions for the cycle selection procedure described in this study had to be optimized. *Hpa* II was used to generate the DNA fragments, since *Hpa* II cutting sites statistically occur every 90 bp in a G+C-rich (65%) organism like *P. aeruginosa*, but are rather absent within A+T-rich Fur-box elements. *Spe* I was chosen as the linker-specific restriction site, and DNA fragments containing an internal *Spe* I site will not be recovered by this procedure, however, there are only very few (≈37) *Spe* I sites within the *P. aeruginosa* chromosome (21). Fragments >0.5 kb did not bind efficiently to the protein G antibody column. Any bias during the PCR of a complex pool of initially about 100,000 individual DNA molecules was avoided by optimizing the pH and the Mg^{2+} concentration in the PCR buffer. The "PCR plateau effect" leading to a preferred amplification of some DNA species was avoided by using low concentrations of dNTPs (0.15 mM each) and using only 25 PCR cycles. False priming was minimized by using low primer concentration (0.5 μM), and inter- or intrafragment priming was suppressed by using <10 ng of template DNA. A Fur concentration of 200 nM ensured proper binding to even weak targets and did not impair subsequent stoichiometric binding of anti-Fur antibodies.

The DNA pool obtained after a few cycles of enrichment contained groups of identical or overlapping fragments; however, DNA sequence comparison revealed some artifacts, most of them transitions of AT to GC base pairs, which could have been caused by errors of *Taq* polymerase during PCR, formation of TT dimers by UV irradiation during gel purification, and depurination in the low pH buffer during the elution from the protein G column. Therefore, more trustworthy DNA fragments to be used for further analysis were isolated from a genomic plasmid library by using the cycle-selected fragments as labeled probes.

A variety of putative target genes of the Fur repressor were isolated by using the cycle selection procedure. Also, several A+T-rich DNA fragments were recovered that interacted with Fur in DNA mobility-shift assays but could not be assigned to be real Fur targets because DNase footprint assays failed and/or RNase protection analyses did not indicate an iron-dependent expression. A 406-bp fragment with only 36% G+C contained three Fur-box-like elements of 63% identity to the consensus, was located at 49.6–52.3′ on the physical map of the *P. aeruginosa* PAO1 genome, and encoded a putative protein with high homology to several RNA-directed DNA polymerases, including

*Hybridization pattern to *Spe* I (S) and *Dpn* I (D) fragments is indicated, and position on the 75′ map of the *P. aeruginosa* PAO1 chromosome is given.

†Nucleotides matching those of the consensus Fur-box are shown in uppercase letters. In the cases of tandemly organized or overlapping Fur-boxes, both are indicated.

‡The number of palindromic pairs of nucleotides within the two half-sites is given.

§The Fur concentration needed for a complete mobility shift was determined.

¶RNAs from cells grown under low and high iron conditions were compared by RNase protection of PIG transcripts.

**Homologies are given as percentages of identity (id.) over the entire amino acid sequence (overall) or over an amino acid sequence of the indicated length (aa). Homologies were calculated as quotient of identical residues to total residues from a Higgins alignment with gap penalties of 10.

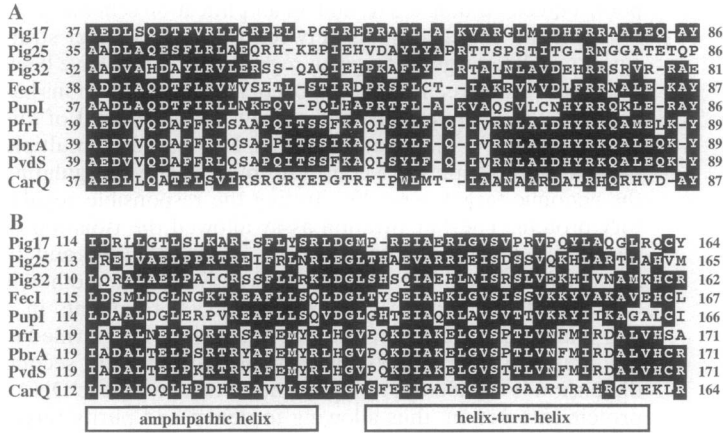

FIG. 4. Alignment of the gene products of *pig17*, *pig25*, and *pig32* with members of the ECF subfamily of bacterial σ factors. Region 2 (*A*), involved in DNA melting, and region 4 (*B*), containing an amphipathic helix and a helix-turn-helix motif (boxed) at the indicated amino acid positions, are shown, and conserved residues are highlighted. Considered as similar are D and E; N and Q; R and K; S and T; F, Y, and W; and I, L, V, and M. The compilation includes only σ factors which are iron regulated, namely *E. coli* FecI (37), *Pseudomonas putida* PupI (38) and PfrI (39), *Pseudomonas fluorescens* PbrA (40), *P. aeruginosa* PvdS (27), and *Myxococcus xanthus* CarQ (41).

the retron-encoded reverse transcriptase responsible for the production of multicopy single-stranded DNA in *E. coli* and *M. xanthus* (52). Another DNA fragment, mapping at 0.3–0.9′, had a A+T content of 61% over a total length of 392 bp and contained a Fur-box-like element of 74% identity to the consensus within an ORF encoding a protein with >50% identity to numerous prokaryotic and eukaryotic serine/threonine kinases, including the *Streptomyces coelicor* PkaA protein kinase (53).

A total of 16 novel genes gave rise to specific DNase footprints with Fur and were expressed during iron starvation. For some of them, interesting sequence homologies to bacterial proteins with a known function were found. Pig12–ORF1 and Pig31, which exhibit some degree of homology to bacterial siderophore receptors, should be analyzed whether they are involved in siderophore-mediated iron uptake. It has been reported that *P. aeruginosa* is extremely versatile in utilizing different types of siderophores from homologous and heterologous sources, including salicylate (54), citrate (55), and numerous fungal compounds (56). In addition, it has been suggested that two distinct uptake systems for ferripyoverdine and ferrienterobactin may exist in *P. aeruginosa* (57, 58). Pig17, Pig25, and Pig32, which share extensive amino acid sequence homology to alternative σ factors, require further studies to determine whether they function as positive regulatory proteins and what their target genes are.

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