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RegA, Iron, and Growth Phase Regulate Expression of the *Pseudomonas aeruginosa tol-oprL* Gene Cluster

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The *tol-oprL* region in *Pseudomonas aeruginosa* appears to be involved in pyocin uptake and required for cell viability. The complete nucleotide sequences of the *tolQRA* and *oprL* genes as well as the incomplete sequences of *tolB* and *orf2* have been previously reported. In addition, the sequence of a *P. aeruginosa* iron-regulated gene (*pig6*) has been described and found to share homology with an open reading frame located upstream of the *Escherichia coli tolQRA* genes (U. A. Ochsner and M. L. Vasil, Proc. Natl. Acad. Sci. USA 93:4409–4414, 1996). In this study, we cloned the remainder of the *P. aeruginosa tol-oprL* gene cluster and determined its nucleotide sequence. This cluster was found to consist of seven genes in the order *orf1 tolQ tolR tolA tolB oprL orf2*. Transcriptional analysis of this gene cluster was performed by detecting the presence of mRNAs spanning adjacent genes as well as by using a promoterless *lacZ* reporter gene fused to each of the seven genes contained in the *tol-oprL* locus. The results show that there are three major transcriptional units or operons in this region, *orf1-tolQRA*, *tolB*, and *oprL-orf2*, in contrast to the *E. coli tol-pal* region, where there are only two operons, *orf1-tolQRA* and *tolB-pal-orf2*. Analysis of gene expression indicated that the *tol-oprL* genes of *P. aeruginosa* are both iron and growth phase modulated. The first operon, *orf1-tolQRA*, is iron regulated throughout growth, but iron-regulated expression of *tolB* and *oprL* fusions occurs only in late log phase. The expression of the three operons was significantly less repressed by iron in *fur* mutants than in the wild-type strain, suggesting the involvement of Fur in the iron regulation of all three operons. RegA is a positive yet nonessential regulator of *tol-oprL* expression.

The Tol system is one of two systems that are involved in macromolecule transport across the outer membrane of gram-negative bacteria. It has been shown that most group A colicins and filamentous phages gain entry into cells through this system in *Escherichia coli* (5, 48), and evidence has been obtained that *tolQ*, *tolR*, and *tolA* are involved in the transport of pyocin in *Pseudomonas aeruginosa* (9). Roles other than membrane transport, such as maintenance of outer membrane integrity, have also been assigned to the Tol-Pal complex. Mutations in the *tol-pal* genes cause the release of periplasmic contents (24, 49) and formation of vesicles (3). Tol-Pal proteins constitute one complex in the inner membrane and one near the outer membrane, and they have been proposed to form a contact site between outer and inner membranes which in turn may mediate interactions between the two membranes (4, 13). Both *tolB* and *tolA* interact with outer membrane porins, possibly affecting either porin assembly (39) or porin activity (24). Evidence suggests that *tolA* may also play a role in positioning the cell division sites since cell division in low- or high-osmolarity medium is impaired in *tolA* mutants (31). The Tol-Pal system in *E. coli* has recently been shown to consist of seven genes organized as two operons, *orf1-tolQRA* and *tolB-pal-orf2* (47).

P. aeruginosa is an important human pathogen capable of causing a diverse range of infections in humans, especially in immunocompromised and cystic fibrosis patients (51). We have previously reported the cloning of the *tolQRA* genes from *P. aeruginosa* (9) and demonstrated that it was not possible to construct isogenic mutants in either *tolQ* or *tolA*, suggesting an essential role for these genes in *P. aeruginosa*. The *oprL* gene (*pal* in *E. coli*) has also been described in *P. aeruginosa* and *P. putida* (28, 40). The sequences of portions of *tolB* have previously been determined (9, 28). A DNA fragment encoding an iron-regulated gene (*pig6*) that exhibits high homology to *E. coli orf1* in the *orf1-tolQRA* operon was isolated as a DNA fragment bound by the *P. aeruginosa* ferric uptake regulator (Fur) (33).

In *E. coli*, the expression of *tolQRA* is regulated by RcsC, a sensor protein in a two-component regulatory system controlling capsule synthesis, possibly through an unidentified mediator (7). The only environmental factor shown to affect *tol-pal* gene expression in *E. coli* was temperature (7). In contrast, we have shown that the expression of *tolQ* and *tolA* in *P. aeruginosa* is iron regulated and that growth temperature also affects expression of these genes (23). However, it was not clear whether the observed iron regulation of these genes in *P. aeruginosa* was dependent on interaction between *orf1* promoter and Fur or other mediators. The effects of iron on other genes in the *tol-pal* cluster had not been determined. In this study, we further examined the genetic organization of the *tol-oprL* cluster in *P. aeruginosa* and determined that there are three major transcriptional units or operons in this region. All three operons were found to be iron regulated, and their expression was modulated during different phases of growth. In addition, we have shown that RegA, a transcriptional activator involved in exotoxin A production (16, 18), appears to positively regulate *tol-oprL* expression in *P. aeruginosa*.

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MATERIALS AND METHODS

Strains, plasmids, primers, and culture conditions. Bacterial strains, plasmids, and oligonucleotide primers used are described in Table 1. *E. coli* strains were

TABLE 1. Bacterial strains, plasmids, and primers

Strain, plasmid, or primer	Relevant properties	Source or reference
E. coli DH5α	φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 endA recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1*	Life Technologies
P. aeruginosa		
PAO1	Prototroph	21
PAO1 C6	*fur* mutant of PAO1	2
PAO1 Δ*pvdS*	PAO1 with deletion of 460 bp of *pvdS*, Gm^r cassette in *pvdS* gene	32
PAO6609	*met-9011 amiE200 rpsL pvd-9*	20
PAO6609 *pchR*	PAO6609 *pchR*::Ω-Tc	17
PA103	Prototroph	30
PA103Δ*regAB::Gm*	PA103 with 1.8-kb *Eco*RV fragment containing *regAB* deleted and replaced with 1.6-kb *Eco*RV Gm^r cassette	38
Plasmids		
pHP45Ω	Source of Ω fragment (Sm^r/Sp^r with transcriptional termination signals in both orientations), Amp^r	36
pNOT19	Cloning vector, Amp^r	42
pNOT8.0	pNOT19 carrying 8.0-kb *Sph*I chromosomal fragment (*orf1-tolQRAB*, *oprL*, and *orf2*), Amp^r	This study
pPA3.5	pUC19 carrying 3.5-kb *Sph*I-*Pst*I fragment (*orf1-tolQRA* and partial *tolB*)	9
pPA3.5RK	pRK415 carrying the 3.5-kb *Sph*I-*Pst*I fragment	9
pRK415	Cloning vector; Tc^r	22
pRKIz	*orf1*::*lacZ* fusion, pRK415 carrying PCR amplified *orf1* P1 region oriented opposite Plac and *lacZ*-Gm^r cassette from pZ1918 inserted in *Bam*HI site; Tc^r, Gm^r	This study
pRKQzT	*tolQ*::*lacZ* fusion, pPA3.5RK with *lacZ*-Gm^r cassette inserted in the *Bgl*II site of *tolQ*, *Hin*dIII fragment containing the terminator from pHP45Ω inserted in the *Hin*dIII site downstream of Plac; Tc^r, Gm^r	This study
pRKQz(ΔP1)T	*tolQ* (ΔP1)::*lacZ* fusion, pRK415 carrying 0.7-kb *Xba*I-*Bgl*II fragment (only coding region of *orf1* and 5′ portion of *tolQ*) in *Xba*I-*Bam*HI site with *lacZ*-Gm^r cassette inserted in the *Kpn*I site, *Hin*dIII Ω fragment in *Hin*dIII site downstream of Plac; Tc^r, Gm^r	This study
pRKRz	*tolR*::*lacZ* fusion, pRK415 carrying 1.9-kb *Sph*I-*Sal*I fragment (*orf1-tolQ* and partial *tolR*), *lacZ*-Gm^r cassette in *Sal*I site, *Hin*dIII Ω fragment downstream of Plac; Tc^r, Gm^r	This study
pRKRz (ΔP1)	*tolR* (ΔP1)::*lacZ* fusion, pRK415 carrying 0.6-kb *Bgl*II-*Sal*I fragment (partial *tolQ* and partial *tolR*, but no P1 or *orf1*, oriented opposite Plac) in *Bam*HI-*Sal*I site with *lacZ*-Gm^r cassette in *Sal*I site; Tc^r, Gm^r	This study
pRKAzT	*tolA*::*lacZ* fusion, pPA3.5RK with *lacZ*-Gm^r cassette in *Xho*I site of *tolA*, *Hin*dIII Ω fragment inserted downstream of Plac; Tc^r, Gm^r	This study
pRKAz (ΔP1)T	*tolA* (ΔP1)::*lacZ* fusion, same as pRKAzT with the *Sph*I-*Sal*I fragment (P1, *orf1*, and 5′ portion of *tolQ*) deleted; *Hin*dIII Ω fragment inserted in *Hin*dIII site downstream of Plac; Tc^r, Gm^r	This study
pRKBzT	*tolB*::*lacZ* fusion, pPA3.5RK with *lacZ*-Gm^r cassette in *Pst*I site, *Hin*dIII Ω fragment downstream of Plac; Tc^r, Gm^r	This study
pRKBz(ΔP1)	*tolB* (ΔP1)::*lacZ* fusion, pRK415 carrying 1.1-kb *Xho*I-*Pst*I fragment (partial *tolA* and partial *tolB*, no P1, *orf1* or *tolQ*, oriented opposite Plac) in *Sal*I-*Pst*I site, *lacZ*-Gm^r cassette in *Kpn*I site; Tc^r, Gm^r	This study
pRKPz (ΔPb)	*oprL*(ΔPb)::*lacZ* fusion, pRK415 carrying 1.2-kb *Pst*I fragment (partial *tolB* and partial *oprL*, oriented opposite Plac) with *lacZ*-Gm^r cassette in *Pst*I site; Tc^r, Gm^r	This study
pRKIIz	*orf2*::*lacZ* fusion, pRK415 carrying 1.5-kb *Pst*I-*Xho*I fragment (partial *tolB*, intact *oprL*, and partial *orf2*) with *lacZ*-Gm^r cassette inserted in *Bam*HI site, *Hin*dIII Ω fragment downstream of Plac; Tc^r, Gm^r	This study
pRKIIz (ΔPp)	*orf2* (ΔPp)::*lacZ* fusion, pRK415 carrying 0.4-kb *Pst*I fragment (3′ portion of *oprL*, 5′ portion of *orf2*) in *Pst*I site with *lacZ*-Gm^r cassette in *Sph*I site; Tc^r, Gm^r	This study
pRKlacT	pRK415 with *Hin*dIII Ω fragment downstream of Plac and *lacZ*-Gm^r cassette inserted in *Sal*I site in the same orientation as Plac; Tc^r, Gm^r	This study
pRKlacO	pRK415 with the *lacZ*-Gm^r cassette inserted in *Sal*I site in orientation opposite Plac; Tc^r, Gm^r	This study
pUC19	Cloning vector, Amp^r	Life Technologies
pZ1918	Vector, source of the cassette containing promoterless *lacZ* and Gm^r (*lacZ*-Gm^r)	43
Primers		
RT-PCR[a]		
orf1/*tolQ*-f	CGGACACCCTGAAGCCAC	
orf1/*tolQ*-r	GCCCTCCATCACCGCATC	
tolQ/*tolR*-f	ACAACCGCTTCTCCGCAC	
tolQ/*tolR*-r	CCAACCACCGCACCATAG	
tolR/*tolA*-f	ATGACCGATGCAGTCACC	
tolR/*tolA*-r	GCCTTCTTTTGTTCCGCC	
tolA/*tolB*-f	GCGGAAGCGGCGAAGAAG	
tolA/*tolB*-r	GCGATGGGAATGGCACGG	
tolB/*oprL*-f	CTAATCTACGCCACCCGCC	
tolB/*oprL*-r	CGCTGACCGCTGCCTTTC	

Continued on following page

TABLE 1—*Continued*

Strain, plasmid, or primer	Relevant properties	Source or reference
oprL/orf2-f	ACAGCTCCGACCTGAAGCC	
oprL/orf2-f	ACAGCTCCGACCTGAAGCC	
oprL/orf2-r	ACCTGCCGTGCCATACCC	
*orf2*dr[b]	CGAAATCCCGGAAGGTCTC	
Amplification of *orf1* P1		
Forward	GAGCGAGGAGCGGCACAC	
Reverse	TCCGAGCCCGTTCCATGAACTTG	

[a] The primers used to test the region between two specific genes are represented by the names of the two genes; forward (upstream) primer and reverse (downstream) primer are indicated by "f" and "r," respectively.
[b] The reverse primer located downstream of *orf2*.

routinely grown in Luria-Bertani (LB) broth or maintained on LB agar plates. *P. aeruginosa* strains were routinely maintained on M9-glucose agar plates or LB agar plates. Bacterial cultures were grown at 37°C with agitation at 220 rpm. Microaerobic conditions were achieved by incubating cultures statically in anaerobic jars with Anaerocult C packs from Merck & Co. (Whitehouse Station, N.J.). Antibiotics were added to the growth media at the following concentrations where appropriate: for *E. coli*, gentamicin at 15 μg/ml, ampicillin at 50 μg/ml, or tetracycline at 15 μg/ml; for *P. aeruginosa* gentamicin at 250 μg/ml or tetracycline at 200 μg/ml. All reagents and media were prepared with H_2O purified by the Milli-Q system (Millipore, Bedford, Mass.).

DNA manipulations. Molecular biology techniques were generally performed as described by Sambrook et al. (41). Restriction enzymes, agarose, DNA size markers, and *Taq* DNA polymerase were purchased from Gibco-BRL (Burlington, Ontario, Canada). T4 DNA ligase was purchased from Promega (Madison, Wis.). DNA fragments were purified from agarose gels with Gene-Clean II (Bio/Can Scientific, Mississauga, Ontario, Canada). Plasmids were introduced into *E. coli* and *P. aeruginosa* by electroporation using a Gene Pulser electroporater (Bio-Rad, Richmond, Calif.) as previously described (10, 41). PCR products were cloned into pCR2.1-TOPO vector as recommended by the manufacturer (Invitrogen, Carlsbad, Calif.).

Isolation of *tolA* downstream region. Chromosomal fragments that overlapped the 3.5-kb fragment containing *orf1-tolQRA* and partial *tolB* (9) were obtained from *Sph*I or *Xho*I digests of chromosomal DNA of *P. aeruginosa* PAO, isolated as previously described (1), and fractionated on sucrose gradients (41). Fractions were hybridized with the 676-bp *Xho*I-*Kpn*I fragment internal to *tolA* (Fig. 1). The probe was labeled with [^{32}P]dCTP by random priming using an Oligolabelling kit from Amersham Pharmacia Biotech (Baie d'Urfé, Québec, Canada) according to the manufacturer's recommendations. The chromosomal DNA fragments that hybridized with the *tolA* probe were cloned into pUC19 or pNOT19 (42).

Nucleotide sequencing and sequence analysis. The nucleotide sequences of *orf1*, *tolB*, and *orf2* were determined by using the ABI Prism DyeDeoxy termination cycle sequencing system with AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, Conn.) and an ABI 1371A DNA sequencer by the University Core

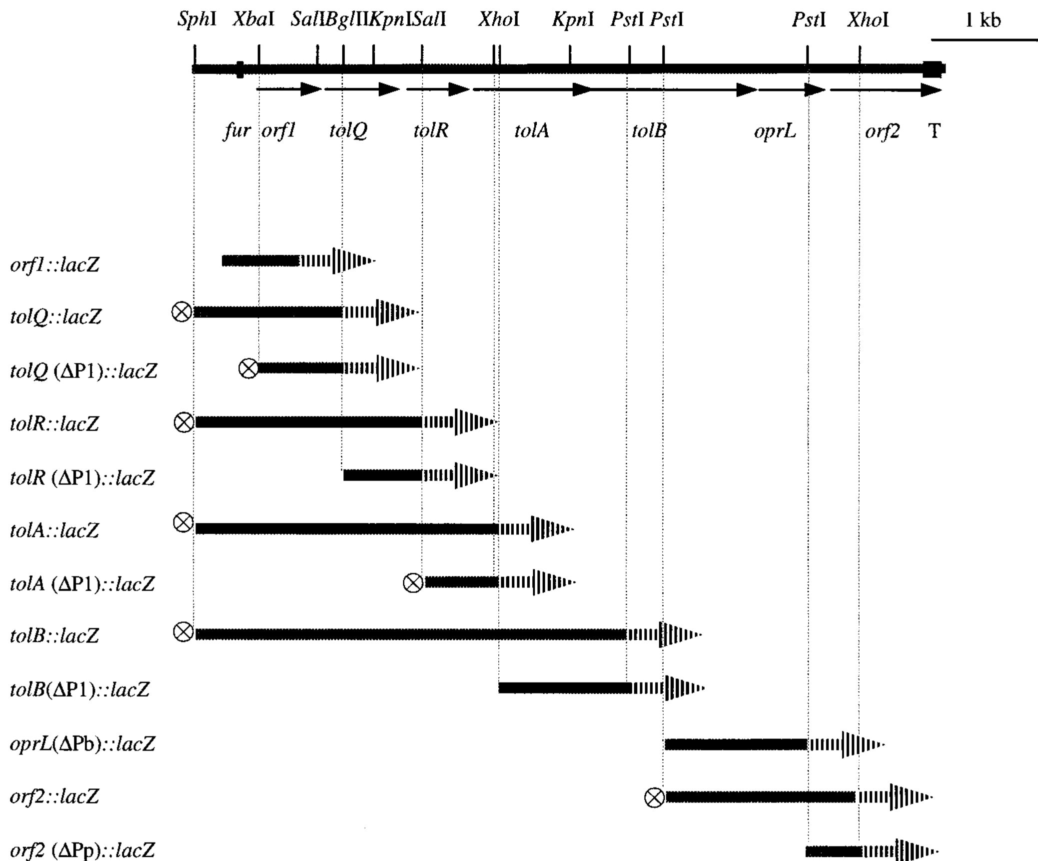

FIG. 1. Genetic organization of *tol-oprL* cluster and *lacZ* fusion constructs of the *tol-oprL* genes. DNA fragments of the *tol-oprL* region are shown as solid bars. The relevant restriction sites, *fur* box, and putative terminator are indicated. The promoterless *lacZ* gene is shown by the arrowhead bars; the transcriptional terminator from pHP45Ω is represented by a circled cross. Only the fragments upstream of *lacZ* are shown in the diagram of the fusion constructs.

DNA Services (University of Calgary). Oligonucleotide primers were synthesized by Gibco-BRL. Analysis of the sequence was performed with PC/Gene software (Intelligenetics, Mountain View, Calif.).

Construction of *lacZ* transcriptional fusions. Transcriptional fusions were constructed by cloning fragments of the *tol-oprL* cluster and a promoterless *lacZ*-Gm^r cassette from pZ1918 (43) into pRK415 (22). The exact fragments and restriction sites used in constructing the fusions are described in Table 1 and Fig. 1. To construct the *orf1::lacZ* fusion, a fragment containing P1 was amplified with the P1 forward and reverse primers (Table 1) and cloned into the *Eco*RI site of pRK415. The *lacZ*-Gm^r cassette was inserted into the downstream *Bam*HI site. The *tolQ::lacZ*, *tolA::lacZ*, and *tolB::lacZ* fusions were constructed from pPA3.5RK by inserting the *lacZ*-Gm^r cassette into the *Bgl*II, *Xho*I, and *Pst*I sites, respectively. The *tolR::lacZ* fusion was constructed by cloning a 1.9-kb *Sph*I-*Sal*I fragment containing partial *tolR* and the upstream region into the *Sph*I-*Sal*I sites of pRK415. The *lacZ*-Gm^r cassette was then cloned into the *Sal*I site. A *Hin*dIII fragment containing transcription terminators from pHP45Ω (36) (*Hin*dIII Ω fragment) was inserted in the *Hin*dIII site downstream of the *lacZ* promoter (Plac) in pPA3.5RK. The *tolQ*(ΔP1)*::lacZ* fusion was constructed by cloning into pRK415 an *Xba*I-*Bgl*II fragment, containing only the coding region of *orf1* and the 5′ portion of *tolQ*, into the *Xba*I-*Bam*HI sites, inserting the *lacZ*-Gm^r cassette in the *Kpn*I site and the *Hin*dIII Ω fragment in the *Hin*dIII site. Similarly, *tolR*(ΔP1)*::lacZ* and *tolB*(ΔP1)*::lacZ* fusions were constructed by first cloning the *Bgl*II-*Sal*I or *Xho*I-*Pst*I fragment, respectively, lacking P1 and *orf1* (Fig. 1) into pRK415 and then inserting the *lacZ*-Gm^r cassette in the *Kpn*I site. To construct the *tolA*(ΔP1)*::lacZ* fusions, pRKAz (23) was first digested with *Sal*I and religated, deleting a *Sal*I fragment (*Sal*I-*Sph*I fragment of the plasmid and *Sph*I-*Sal*I of the *tol-oprL* fragment containing P1 and *orf1*). The plasmid portion was restored by ligating a *Sal*I fragment from pRK415 to the intermediate plasmid. The *oprL*(ΔPb)*::lacZ* fusion was made by inserting a *Pst*I fragment containing the 3′ portion of *tolB* and the 5′ portion of *oprL* and the *lacZ*-Gm^r cassette in the *Pst*I site. The *orf2::lacZ* fusion in pRK415 contains a fragment with part of *tolB*, *oprL*, and part of *orf2* in the *Pst*I-*Sal*I sites, the *lacZ*-Gm^r cassette in the *Bam*HI site, and the *Hin*dIII Ω fragment in the *Hin*dIII site. The *orf2*(ΔPp)*::lacZ* fusion contains a *Pst*I fragment (3′ portion of *oprL*, 5′ portion of *orf2*, and a 6-bp *Sal*I-*Pst*I segment of pNOT19, without Pp, in the opposite orientation to Plac) in the *Pst*I site with the *lacZ*-Gm^r cassette in the *Sph*I site.

β-Galactosidase assay. β-Galactosidase assays were performed as previously described (35). Cultures of *P. aeruginosa* harboring the *tol::lacZ* fusions were grown in TSB-DC broth (34) at 37°C with aeration unless otherwise stated. The iron concentration of TSB-DC has previously been determined to be approximately 1.0 μM (34). Medium was supplemented with either ethylenediamine-di(*o*-hydroxyphenylacetic acid) (EDDHA; 400 μg/ml) or 50 μM $FeCl_3$, and cells grown overnight in TSB-DC were used for inoculation of the cultures for assays. Samples were diluted 1/10 if the optical density at 600 nm of the culture exceeded 1.0. All assays were performed in triplicate. *P. aeruginosa* strains harboring pRKlacT or pRKlacO (Table 1) were used as negative controls.

Total RNA isolation and RT-PCR. Total RNA from *P. aeruginosa* was isolated using a Qiagen RNA Midi kit (Qiagen, Mississauga, Ontario, Canada) from 5 ml of culture at 8 and 20 h of growth at 37°C in TSB-DC with EDDHA at 400 μg/ml. The RNA obtained was treated with amplification-grade DNase I (Gibco-BRL) before use. Reverse transcription-PCR (RT-PCR) was performed using a Titan one-tube RT-PCR kit (Boehringer Mannheim, Mississauga, Ontario, Canada), with minor modifications of the manufacturer's recommendations. Thirty nanograms of total RNA was used in each reaction. The cDNA was synthesized from the downstream primer by reverse transcriptase using RNA as the template. The double-stranded DNA was synthesized and amplified by PCR using both upstream and downstream primers. Reverse transcription was carried out at 50°C for 30 min, and 35 cycles of PCR were performed as follows: 10 cycles of denaturation at 95°C for 30 s, annealing at 60-64°C for 30 s, and elongation at 68°C for 45 s, followed by 25 cycles with increased elongation time in each cycle. An additional 5 s was added to each subsequent cycle; i.e., the 11th cycle has an elongation time of 50 s, and the 12th cycle has an elongation time of 55 s, etc. Amplification was stopped following a final elongation at 68°C for 10 min. The annealing temperature in each reaction was determined according to the composition of the primers used. RT-PCR products were examined by agarose gel electrophoresis. Negative controls used included RNA samples treated with RNase prior to reaction and heat inactivation of reverse transcriptase in Titan one-enzyme mixture before use. DNA contamination of the mRNA was determined by PCR using *Taq* polymerase without reverse transcriptase.

Nucleotide sequence accession numbers. The nucleotide sequences reported here have been deposited in GenBank and assigned accession no. U39558 for *orf1*, *tolQRAB*, and AF177774 for *orf2*.

RESULTS

Organization of the *tol-oprL* genes in *P. aeruginosa*. To complete the analysis of the gene organization of the *tol-oprL* region in *P. aeruginosa*, the regions upstream of *tolQ*, downstream of *tolA*, and downstream of *oprL* were sequenced. The previously cloned 3.5-kb *Sph*I-*Pst*I chromosomal DNA fragment in pPA3.5 (9) was used to sequence the upstream region of *tolQ*. To sequence the entire *tolB* and downstream of the *tol-oprL* cluster, an 8.0-kb *Sph*I fragment and a 2.9-kb *Xho*I fragment, which hybridize with a 676-bp *tolA* probe (data not shown), were isolated from the *P. aeruginosa* chromosome. Sequence analysis confirmed that the *tol-oprL* region of *P. aeruginosa* contains seven genes in the order *orf1 tolQ tolR tolA tolB oprL orf2* (Fig. 1). The stop codon of *orf1* is separated from the start codon of *tolQ* only by 1 bp, suggesting possible translational coupling. The distance between *tolQ* and *tolR* is 22 bp, and that between *tolR* and *tolA* is only 2 bp. The distances present between *tolA* and *tolB* and between *tolB* and *oprL* are 38 and 49 bp, respectively. *orf2* is separated from *oprL* by 9 bp. Fifty-two base pairs upstream of *orf1* is a gene (*ruvB*) encoding Holliday junction-specific DNA helicase (19) and downstream of *orf2* is a potential rho-independent transcriptional terminator, indicating that both boundaries of the gene cluster have been reached. As reported previously (33), the promoter region of *orf1* has a *fur* box with 12 out 19 bp identical to the consensus sequence. The organization of the seven genes in the *P. aeruginosa tol-oprL* cluster is almost identical to that observed for the *E. coli tol-pal* gene cluster, although the latter has not been reported to contain a *fur* box.

orf1 potentially encodes a polypeptide of 148 amino acid residues with a molecular mass of 16.7 kDa that shares no homology with any protein of known function in GenBank. Directly following *tolA* is *tolB*, which encodes a predicted protein of 47.8 kDa. The first 21 amino acid residues at its N terminus form a potential secretory signal sequence. The predicted TolB protein is 78.7% identical to *P. putida* TolB and shares 44.2 and 40.3% identity with *E. coli* TolB (27) and *Haemophilus influenzae* TolB (44), respectively. *orf2* potentially encodes a polypeptide of 275 amino acid residues with a molecular mass of 29.1 kDa, which is similar in size to the Orf2 of 262 amino acid residues in *E. coli*. A computer-predicted secretory signal sequence is also present at the N terminus of Orf2 with two alternative processing sites between residues 19 and 20 or between residues 21 and 22. The *orf2* sequence of *P. aeruginosa* previously reported by Lim et al. suggests that *orf2* encodes a protein of 107 residues and is followed by an insertion sequence (28). The *orf2* sequence obtained from our PAO strain did not contain an insertion sequence.

Transcriptional analysis of the *tol-oprL* cluster. To examine the operon structures of the *tol-oprL* cluster in *P. aeruginosa*, transcriptional fusion analysis was used. mRNA analysis has been shown to be difficult in studying the *tol-oprL* transcription in both *P. aeruginosa* (9, 23) and *E. coli* (47). Both Northern hybridization and primer extension proves to be problematic presumably due to the low abundance or instability of the transcripts. Therefore, a promoterless *lacZ* reporter gene was fused to the *tol-oprL* genes cloned on a low-copy-number plasmid, pRK415 (Fig. 1). The *lacZ* was fused in each of the seven genes containing only the individual gene's upstream region to test whether each gene has its own promoter. Fusions were also constructed with the presence of intact upstream genes and their potential promoter regions to test the expression directed by upstream promoters. A transcriptional terminator from pHP45Ω (36) was used in some of the constructs to eliminate the possible effect of the vector-encoded Plac when the gene under study was in the same orientation as Plac. No residual promoter activity was observed from the vector when the Plac promoter was in the opposite orientation to the fusion. The expression of these fusions was determined by measuring β-galactosidase activity.

As shown in Table 2, high levels of expression independent of the upstream genes were observed only with the *orf1*, *tolB*,

TABLE 2. β-Galactosidase activity of PAO1 containing the *tol*::*lacZ* fusion constructs

Fusion construct	β-Galactosidase activity (Miller units)[a]
orf1::lacZ	10,560 ± 251
tolQ::lacZ	23,148 ± 358
tolQ (ΔP1)*::lacZ*	870 ± 35
tolR::lacZ	19,157 ± 296
tolR (ΔP1)*::lacZ*	353 ± 11
tolA::lacZ	14,739 ± 445
tolA (ΔP1)*::lacZ*	1,655 ± 34
tolB::lacZ	13,292 ± 649
tolB(ΔP1)*::lacZ*	14,115 ± 452
oprL(ΔPb)*::lacZ*	26,053 ± 1301
orf2::lacZ	23,021 ± 796
orf2 (ΔPp)*::lacZ*	158 ± 6
pRKlacT	345 ± 17
pRKlacO	198 ± 39

[a] Means ± standard deviations of triplicate experiments. Samples were taken after 20 h of growth in TSB-DC medium supplemented with EDDHA at 400 μg/ml.

and *oprL* fusions. When *orf1* and its upstream region was present, *tolQ*, *tolR*, and *tolA* were also expressed at high levels. But when the *orf1* region was deleted, *tolR* was expressed only at background levels and the expression of *tolQ* and *tolA* was greatly reduced to levels approximately 9- to 27-fold less than that in constructs containing *orf1* and its promoter region. These data indicate the major transcriptional activity of *orf1-tolQRA* was from the *orf1* promoter (P1). Low levels of expression of *tolQ* and *tolA* in the absence of P1 suggest that there could be also promoters present upstream of these two genes. In contrast to *tolQ*, *tolR*, and *tolA* fusions, the *tolB* fusion lacking the P1 promoter region exhibited a high level of expression as measured by β-galactosidase activity, suggesting that *tolB* itself has a strong promoter (designated as Pb) and that Pb in the absence of P1 is sufficient for strong expression of *tolB*. There was no difference between the expression of *tolB*(ΔP1)*::lacZ* and *tolB::lacZ* (including P1); however, pRKBzT was considerably less stable than pRKBz(ΔP1), which makes it difficult to compare the expression levels between fusions with both the P1 and Pb promoters to the fusion with only the Pb promoter. Despite growth in medium containing gentamicin, 65% of the cells containing pRKBz(ΔP1) lost the plasmid by 20 h of growth, as determined by comparing CFU counts on LB agar with and without gentamicin. In contrast, cultures harboring pRKBzT demonstrated a plasmid loss of only 9% during the same time period. This high level of plasmid instability was not detected with the other fusion constructs examined. The *orf2*(ΔPp)*::lacZ* fusion displayed a background level of expression, indicating that it is part of an operon that includes the upstream *oprL*. Strong expression of *orf2*, however, was observed in the absence of Pb, the *tolB* promoter. The *orf2::lacZ* fusion construct containing *oprL* and its upstream region, but lacking Pb, exhibited a high level of expression (Table 2), indicating the presence of the third major promoter upstream of *oprL* (designated Pp). The presence of Pp was confirmed by the high-level expression of the *oprL*(ΔPb)*::lacZ* fusion, which also lacks Pb. Therefore, there are three major operons in this cluster, consisting of *orf1-tolQRA*, *tolB*, and *oprL-orf2*. The organization of the *tol-oprL* region of *P. aeruginosa* is distinct from that in *E. coli*, where only two operons, *orf1-tolQRA* and *tolB-pal-orf2*, are present (47).

To examine the expression of the *tol-oprL* genes in *P. aeruginosa*, RT-PCR was also used to analyze mRNA isolated from strain PAO. Primers were designed to amplify the junction regions of each pair of adjacent genes from the mRNA template. If adjacent genes were cotranscribed, a PCR product would be generated following the synthesis of cDNA from RNA templates by reverse transcriptase. Otherwise no PCR products would be produced. The RT-PCR was performed on RNA isolated from both log-phase and stationary-phase cultures, and similar results were obtained. The results obtained correlate with the data obtained by analysis of the *lacZ* fusions. PCR products were generated from the primers amplifying the regions between *orf1* and *tolQ*, *tolQ* and *tolR*, *tolR* and *tolA*, and *oprL* and *orf2* (data not shown), demonstrating the members of each pair are in the same operon. No product was obtained from the primers amplifying the region from *orf2* to downstream of the putative rho-independent terminator (primer *orf2*dr and *oprL*/*orf2*-f), showing that no detectable mRNA extended beyond the terminator was present in the RNA isolates. This result also suggests the functionality of the putative transcriptional terminator. PCR products were also obtained, however, from primers amplifying regions between *tolA* and *tolB* and between *tolB* and *oprL*. These data likely indicate the incomplete transcriptional termination between the major transcriptional units, resulting in transcripts containing the whole cluster and transcripts containing *tolB*, *oprL*, and *orf2*. Similar residual upstream promoter activity and possible presence of long transcripts have also been observed in the *tol-pal* region of *E. coli* (47).

Iron- and growth phase-modulated expression of the *tol-oprL* operons. Previously *tolQ* and *tolA* fusions containing *orf1* have been shown to be iron regulated during mid-log phase of growth (23). Therefore, experiments were conducted to test possible iron-regulated expression of the other two operons and to examine the iron regulation of all three operons during other phases of growth. Expression of the first operon was monitored by measuring the β-galactosidase activity of *orf1::lacZ*, *tolQ::lacZ*, and *tolA::lacZ*, while β-galactosidase activities of *tolB*(ΔP1)*::lacZ* and *oprL*(ΔPb)*::lacZ* were measured to represent expression of the second and third operons, respectively. Expression of *tolQ*(ΔP1)*::lacZ* and *tolA*(ΔP1)*::lacZ* was also measured to monitor activities of the putative independent promoters upstream *tolQ* and *tolA*, respectively. The expression of these fusions was monitored throughout growth.

As shown in Fig. 2, the expression of all three operons appears to be iron regulated and growth phase dependent; however, differences in both gene expression and iron regulation profile were observed between the operons. In agreement with the observation above that *orf1-tolQRA* are in the same operon, the expression of *orf1::lacZ* (Fig. 2A), *tolQ::lacZ*, and *tolA::lacZ* (data not shown) was iron regulated in a similar manner throughout growth. In iron-rich conditions, expression of β-galactosidase activity was consistently lower than in iron-restricted conditions. In iron-restricted conditions, expression declined during log phase; however, in early stationary phase the expression again increased to maximum levels. The repression level (ratio of expression in iron-restricted to expression in iron-rich conditions) of *orf1::lacZ* was approximately 3.1-fold at 4 h and 4.8-fold at 30 h. The expression levels between iron-restricted and iron-rich medium were significantly different at all time points ($P < 0.001$) (Fig. 2A). In contrast, the expression of *tolB*(ΔP1)*::lacZ* and *oprL*(ΔPb)*::lacZ* (Fig. 2C and D) was also iron regulated, but only when the culture reached the late log phase of growth. β-Galactosidase activity was not iron regulated during the early stage of growth. The repression levels for *tolB*(ΔP1)*::lacZ* and *oprL*(ΔPb)*::lacZ* at 30 h were approximately 1.6- and 1.4-fold, respectively. These levels of repression were reproducible in that similar results

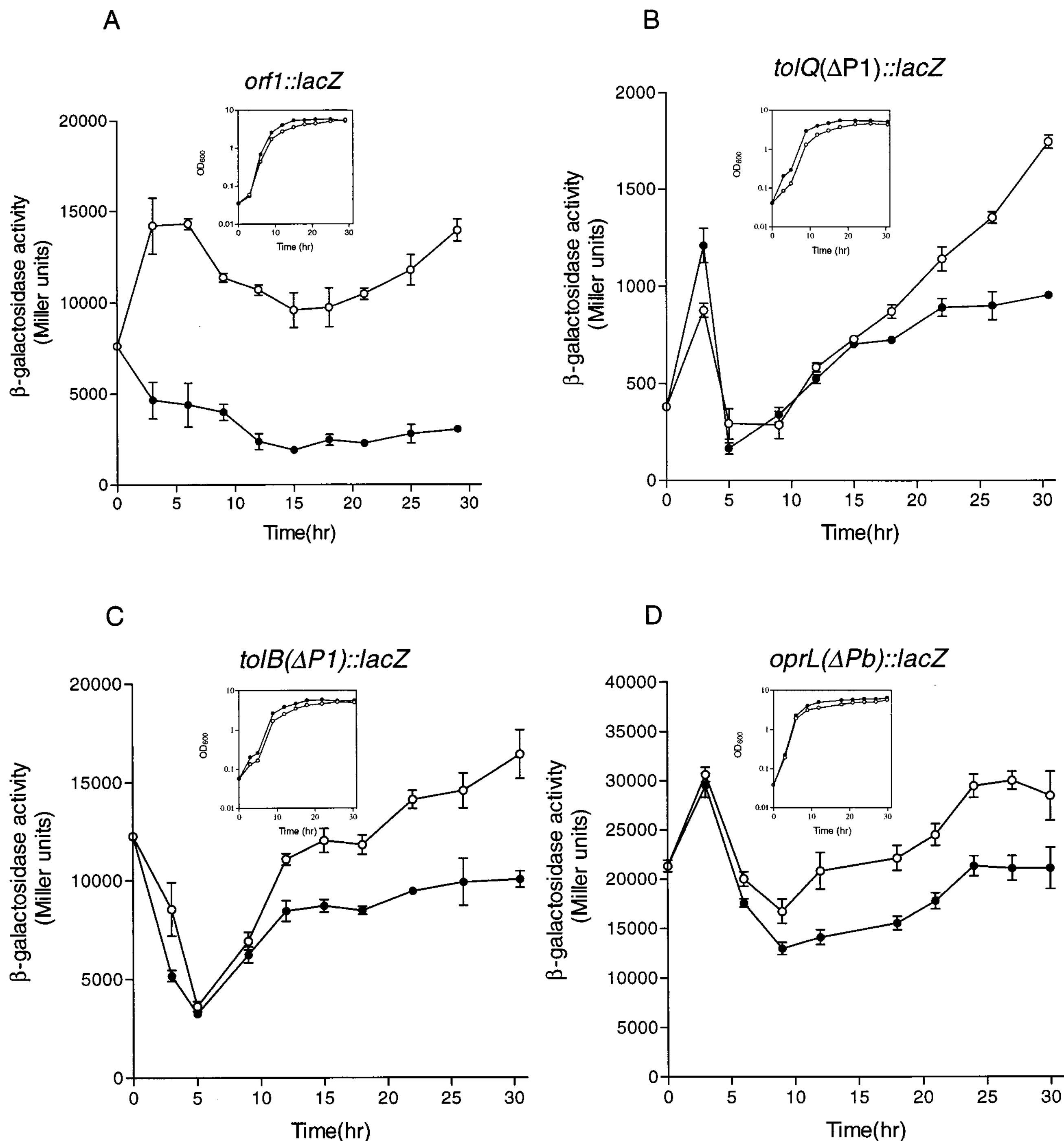

FIG. 2. Effect of iron and growth on expression of the *tol-oprL* genes. Cells were grown in TSB-DC medium supplemented with either 400 μg of EDDHA per ml (open circles) or 50 μM $FeCl_3$ (solid circles). Bacterial growth is shown in the insets. β-Galactosidase activities are means ± standard deviations of triplicate cultures. Similar results were obtained in three independent experiments.

were obtained in three experiments, and the differences in expression between iron-restricted and iron-rich conditions were significantly different ($P < 0.01$) at all time points between 12 and 30 h. The expression of *tolQ*(ΔP1)*::lacZ* (Fig. 2B) and *tolA*(ΔP1)*::lacZ* (data not shown) was similar to *tolB* (ΔP1)*::lacZ* and *oprL*(ΔPb)*::lacZ* in late log and stationary phase and was significantly different between iron-restricted and iron-rich conditions at time points between 18 and 30 h ($P < 0.01$). These results indicate that the expression of the operons in the *tol-oprL* region of *P. aeruginosa* is modulated by both iron and growth phase, but that there are differences in the expression of these operons.

Involvement of Fur in iron regulation of *tol-oprL* expression. Fur has been shown to bind to the *orf1* promoter region (33) and to affect expression of *tolQ::lacZ* or *tolA::lacZ* fusions containing the P1 promoter (23). As *orf1-tolQRA* form one

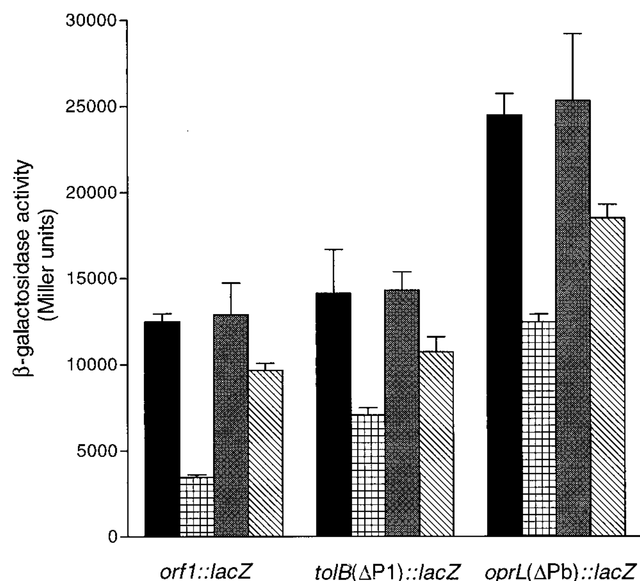

FIG. 3. Effect of *fur* on expression of the three major *tol-oprL* transcriptional units under aerobic conditions. β-Galactosidase activities are means ± standard deviations from triplicate cultures. Samples were taken after 20 h of cultivation in TSB-DC with EDDHA at 400 μg/ml (PAO, black bars; C6, gray bars) or 50 μM $FeCl_3$ (PAO, cross-hatched bars; C6, diagonal-hatched bars). The expression levels of each fusion in C6 were significantly different than in PAO in iron-rich medium ($P < 0.01$, analysis of variance).

operon, it is likely that Fur directly regulates the expression of these genes by binding to the *fur* box in P1. To further examine Fur involvement in the iron regulation of the *orf1-tolQRA*, *tolB*, and *oprL-orf2* operons, the fusions *orf1::lacZ*, *tolB*(ΔP1)*::lacZ* and *oprL*(ΔPb)*::lacZ* were transferred to *fur* mutant C6, which has a point mutation causing a single amino acid residue change (A10→G) (2). Expression was tested in both iron-restricted and iron-rich conditions. The experiments were carried out in both aerobic and microaerobic conditions because it has been shown that Fur may affect gene expression differently in these conditions (2). The expression of the *orf1*, *tolB*, and *oprL* fusions in iron-rich medium in stationary phase was significantly higher in the *fur* mutant than in PAO, whereas the expression of these fusions in iron-restricted medium in PAO was similar to that in C6 (Fig. 3). Similar results were obtained under microaerobic conditions (data not shown). These results indicate that Fur is involved in the iron regulation of *tolB* and *oprL-orf2* as well as *orf1-tolQRA*.

RegA as a positive regulator in *tol-oprL* expression. Although the data indicate the direct involvement of Fur in regulating *orf1-tolQRA* expression, no recognizable *fur* box motifs are present in either the Pb or Pp region. The involvement of Fur in *tolB* and *oprL-orf2* expression is likely to be indirect, possibly through an iron-regulated mediator. The presence of expression peaks of the first operon (Fig. 2A) also suggests there are possibly regulators other than Fur in coordinating its expression. Several known iron-regulated transcriptional regulators including PchR, PvdS, and RegA were examined for their effects on the expression of *tol* genes. PchR is an AraC-type transcriptional activator involved in the synthesis of pyochelin and a ferripyochelin receptor (17). PvdS is an alternative sigma factor that is required for pyoverdine and exotoxin A production (8), and RegA is a transcriptional activator regulating the expression of *toxA* encoding exotoxin A (16, 18). Plasmids containing the *orf1::lacZ*, *tolB*(ΔP1)*::lacZ*, and *oprL* (ΔPb)*::lacZ* fusions were transferred to individual *pchR*, *pvdS*, and *regAB* mutants, and expression was compared to that of the parent strains in both iron-rich and iron-restricted conditions. No significant difference in expression of the three fusions was observed in the *pchR* and *pvdS* mutants compared to PAO (data not shown). The expression of all the three *tol-oprL* operon fusions, however, was affected by the *regAB* mutation (Fig. 4). Compared to the parent strain, the expression of these three operon fusions, and particularly *oprL*(ΔPb)*::lacZ*, was lower in the *regAB* mutant. In iron-restricted conditions, the expression levels of all three fusions in the *regAB* mutant remained relatively constant throughout growth, with only a slight increase in expression during stationary phase. In PA103, the parent strain, there was a significant increase in expression of all three *lacZ* fusions in iron-restricted conditions during stationary phase, similar to that observed with strain PAO (compare Fig. 2 and 4). There also appeared to be a decrease in the level of expression of the *oprL*(ΔPb)*::lacZ* fusion in PA103Δ*regAB::Gm* during log phase in both iron-rich and iron-restricted conditions compared to the parent strain (Fig. 4C). These data suggest that although *regA* is not required for expression of the *orf1-tolQRA*, *tolB*, or *oprL-orf2* operon, it enhances expression of these genes in late log phase of growth under iron-restricted conditions and therefore serves as a positive regulator of *tol-oprL* gene expression.

DISCUSSION

The *tol-oprL* genes play important roles in gram-negative bacteria. The data from this and previous studies indicate that the *tol-oprL* region of *P. aeruginosa* consists of seven genes in the order *orf1 tolQ tolR tolA tolB oprL orf2*. An unrelated DNA helicase is located upstream of *orf1*, and the last gene, *orf2*, is followed by a putative rho-independent transcriptional terminator. This terminator appears to be functional as indicated by RT-PCR analysis in that no transcripts extended beyond the terminator were detectable. Previously, a terminator present within an insertion sequence has been reported to be located downstream of *orf2* (28), but no insertion sequence could be identified in the cloned region in this study. It seems possible that the insertion sequence transposed into the *orf2* region in the PAO isolate used in the study by Lim et al. (28).

Three operons appear to exist in the *tol-oprL* cluster in *P. aeruginosa*. *lacZ* fusion analysis indicates there are three major promoters, P1, Pb, and Pp, upstream of *orf1*, *tolB*, and *oprL*, respectively. It has been shown in *E. coli* that the TolQRA proteins form a complex in the inner membrane via their transmembrane domains (11, 26), and TolB, Pal, and Orf2 are positioned in the outer membrane or periplasm, possibly forming a complex with other outer membrane components (4, 6, 25, 26). In *P. aeruginosa*, it is likely that localization of the Tol-OprL proteins is similar to that in *E. coli*. By being arranged in operons, the major transcriptional activities of these genes may be coordinated but also differentially regulated, which may be important for their functions.

Interestingly, apart from the terminator downstream of *orf2*, no typical terminators could be identified downstream of the first two operons, *tolA* and *tolB*. The lack of obvious terminators makes it possible that the transcription from P1 could also read through *tolB* and *orf2*, and the transcription from Pb could read through *oprL* and *orf2*. The results from RT-PCR analysis support this possibility since mRNA spanning the regions between *tolA* and *tolB* and between *tolB* and *oprL* could be detected from the total RNA isolates. A similar phenomenon was also observed in *E. coli*, where the *orf1* promoter is able to direct the transcription of the whole *tol-pal* cluster (47).

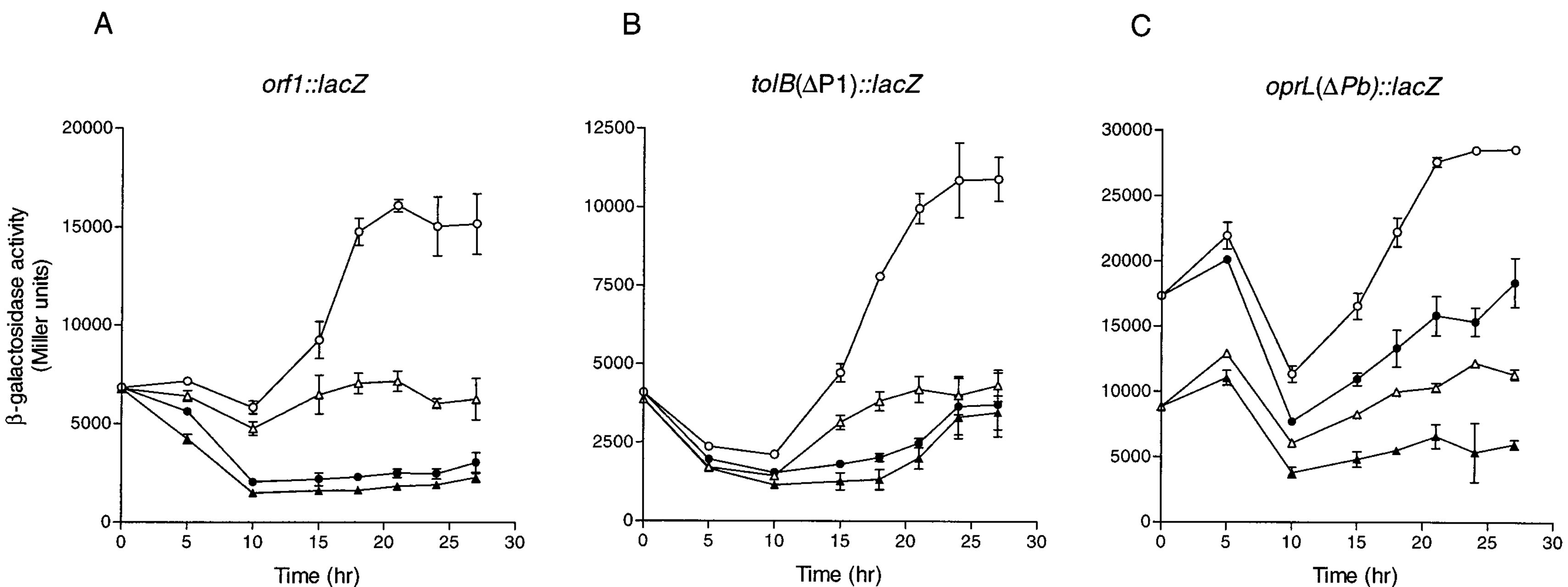

FIG. 4. Effect of *regA* on expression of the *tol-oprL* operons. PA103 carrying *lacZ* fusion constructs was grown in TSB-DC medium supplemented with either 400 μg of EDDHA per ml (open circles) or 50 μM $FeCl_3$ (solid circles). PA103Δ*regAB::Gm* carrying *lacZ* fusion constructs grown in TSB-DC medium supplemented with either 400 μg of EDDHA per ml or 50 μM $FeCl_3$ is represented by open or solid triangles, respectively. β-Galactosidase activities are means ± standard deviations from triplicate cultures. Similar results were obtained in three experiments.

In addition to the three major promoters in the *P. aeruginosa tol-oprL* region, weak promoter activity was also observed upstream of *tolQ* and *tolA*. This transcriptional organization may ensure a minimum expression of each gene product to perform essential functions.

Previously, using Northern hybridization analysis, we detected transcripts of approximately 1.5 kb using *tolQ* and *tolR* probes and an approximately 1.2-kb transcript using a *tolA* probe (9). A potential transcriptional start site was detected upstream of the *tolA* gene, using primer extension analysis (23). These data suggested that *tolQ* and *tolR* were cotranscribed and *tolA* was transcribed separately. In light of the current data obtained with *lacZ* reporter fusions and RT-PCR, it is likely that the transcripts detected in the previous studies (9, 23) were the result of endonucleolytic cleavage of the mRNA.

Iron-regulated expression of the *tol-oprL* genes in *P. aeruginosa* is somewhat unique. There are no reports suggesting that expression of the *tol-pal* genes of *E. coli* or *H. influenzae* is iron regulated, although this possibility may not have been investigated. All three operons in the *tol-oprL* cluster of *P. aeruginosa* displayed iron-regulated expression, although differences were observed during different stages of growth.

Expression of *orf1-tolQRA* was iron regulated throughout growth. The presence of 50 μM $FeCl_3$ in the medium resulted in at least 50% reduction in expression compared to iron-restricted medium. Fur has been shown to play a central role in iron regulation in gram-negative bacteria (29, 33). In the presence of iron, Fur and cytoplasmic Fe^{2+} form a complex and bind to the *fur* box in the promoters; hence, transcription of the iron-regulated genes is repressed. In the absence of iron, Fur does not bind to the *fur* box and expression proceeds (29). Since binding of Fur-Fe^{2+} to the *orf1* promoter has been demonstrated (33), it is clear that the iron regulation of these genes directly involves Fur. These data are confirmed by the observation that the repression of *orf1* expression by iron is significantly decreased in *fur* mutants, where Fur-Fe^{2+} binding capacity is less efficient than for the wild-type complex (2).

Expression of *orf1-tolQRA* was iron regulated throughout growth; however, iron regulation of *tolB* and *oprL-orf2* expression was detected only in late log to early stationary phase of growth. The decreased iron repression in the *fur* mutant indicated that Fur is also involved in the regulation of *tolB* and *oprL-orf2* expression; however, such an involvement seems to be indirect. A search for *fur* boxes in the *tolB* and *oprL* promoter regions failed to identify any such motifs. Presence of an intermediate regulator, by which Fur may regulate the mediator and the mediator in turn would modulate the expression of the *tol-oprL* operons, was therefore postulated. This kind of hierarchy in iron regulation has been shown to be common in *P. aeruginosa* (46).

Expression of the *orf1::lacZ*, *tolB*(ΔP1)*::lacZ*, and *oprL* (ΔPb)*::lacZ* fusions was less iron regulated in the *fur* mutant C6 than in PAO (Fig. 3). Similar results were previously shown with *tolQ::lacZ* and *tolA::lacZ* fusions with the P1 promoter in two other fur mutants, A2 and A4 (2, 23). There was no difference in *fur* regulation between cultures grown in either aerobic or microaerobic conditions. Siderophore production, detected by chrome azurol S activity, was reported to be constitutive in C6 in high-iron medium regardless of the oxygen levels of the medium (2). Exotoxin A yields, however, were deregulated only in high-iron microaerobic conditions in the C6 mutant. RegA transcription was also shown to be constitutive in microaerobic but not aerobic conditions in C6 (2). Although RegA also enhances *tol* gene expression, Fur may regulate *tol* gene expression in a more similarly to siderophore biosynthesis gene expression than to exotoxin A or *regA* expression, since similar results were obtained with C6 in both aerobic and microaerobic conditions.

The transcriptional activator RegA was found to increase the expression of the *tol-oprL* genes in stationary phase in iron-restricted medium. In PA103, *regA* has been shown to have two promoters, P1 and P2, which direct the synthesis of the T1 and T2 transcripts, respectively. The T1 transcript encodes both *regA* and *regB*, while the T2 transcript encodes only *regA* (45). The *regAB* P1 promoter is not significantly affected by iron; however, the *regA* P2 promoter is iron regulated (45). P2 activity starts rising in late log to early stationary phase (45), which corresponds to the peak expression of *orf1-tolQRA* and the beginning of iron regulation of *tolB* and *oprL-orf2*. Although the mutation in PA103Δ*regAB::Gm* also eliminates the expression of *regB*, alterations in *tol* gene expression in this mutant are most likely due to the loss of *regA* since the iron-regulated expression of the *tol* genes more closely parallels the expression of the T2 transcript. RegA appears to be required for increased expression of the *tol-oprL* genes in iron-restricted medium, but it is not essential for the expression of these genes. In iron-rich medium, expression of the *tol-oprL* operons was also decreased in the *regAB* mutant compared to the parent. Although the decrease in expression observed with the *orf1* and *tolB* fusions between the mutant and the parent was small, it was reproducible and significantly different between 5 and 21 h for *orf1::lacZ* and 5 and 24 h for *tolB*(ΔP1)*::lacZ* ($P < 0.05$). The difference observed in *oprL*(ΔPb)*::lacZ* expression between the two strains was significant throughout growth ($P < 0.005$). There is a level of iron-regulated expression of the *tol-oprL* genes in *P. aeruginosa* that is not due to either Fur-Fe^{2+} or RegA, suggesting that other potential regulators are involved in the expression of *tol-oprL* genes. It is not surprising to observe some iron regulation of the *orf1-tolQRA* operon in the *regAB* mutant because Fur would regulate *orf1* P1 in this genetic background. But since Fur does not appear to regulate the Pb or Pp promoter directly, the iron regulation of these operons in the *regAB* mutant suggests the presence of other regulatory factors.

RegA is one of several factors that regulates the synthesis of exotoxin A. Other factors that influence exotoxin A expression include LasR (12), Fur (37), Vfr (50), PvdS (32), and PtxR (14). With the exception of *lasR*, these genes also regulate *regA* expression. PtxR and Vfr increase *regA* transcription through the P1 promoter (14, 50). PvdS activates expression of both the T1 and T2 transcripts in strain PAO (32). Although the effects of Vfr and PtxR on *tol-oprL* expression were not examined, it is possible that they would also enhance expression of the *tol-oprL* genes due to their roles in *regA* expression. Since *pvdS* was shown to be required for the detection of *regA* transcripts in PAO (32), it was somewhat surprising that there was no difference in the expression of the *tol* operon fusions in the *pvdS* mutant compared to PAO. Although neither *regA* nor *toxA* transcripts were detectable in this mutant, low levels of exotoxin A were detected in culture supernatants by immunoblotting (32). This suggests that low levels of *regA* were also expressed. The inability of these investigators to detect *regA* was likely due to the insensitivity of the assay and the short half-lives of the transcripts (32). The amount of RegA produced in the *pvdS* mutant may be sufficient for enhancement of *tol-oprL* gene expression.

The PA103 *regAB* mutant does not produce exotoxin A (38) yet still expresses the *tol-oprL* genes, although at lower levels than the parent strain. The mechanism by which RegA activates *toxA* expression, however, is not clear. RegA shares little homology with other known transcriptional regulators, and

binding of RegA to the *toxA* promoter could not be demonstrated in mobility shift assays (15). Unlike other transcriptional regulators, RegA has been proposed to interact specifically with RNA polymerase prior to association with the promoter DNA (48). Further studies are needed to determine whether RegA activates *toxA* and *tol-oprL* gene expression in similar manners.

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