JOURNAL OF BACTERIOLOGY, Feb. 1999, p. 1099–1109 Vol. 181, No. 4
0021-9193/99/$04.00+0
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Pseudomonas aeruginosa fur Overlaps with a Gene Encoding a Novel Outer Membrane Lipoprotein, OmlA

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Received 8 September 1998/Accepted 9 December 1998

A novel outer membrane lipoprotein in *Pseudomonas aeruginosa* is encoded by the *omlA* gene, which was identified immediately upstream of the *fur* (ferric uptake regulator) gene. The *omlA* and *fur* genes were divergently transcribed and had overlapping promoter regions. The proximal *fur* P2 promoter and the *omlA* promoter shared a 5-bp DNA motif for their −10 promoter elements. The distal *fur* P1 promoter was located within the *omlA* coding sequence, and the *omlA* and *fur* T1 mRNAs overlapped by 154 nucleotides. Optimal expression of both *fur* and *omlA* required roughly 200 bp of DNA upstream of the promoter regions, suggesting the presence of *cis*-acting transcriptional activation elements located within the *omlA* and *fur* genes, respectively. The levels of Fur and OmlA proteins had no influence on *omlA* or *fur* expression, excluding any *trans*-acting cross-regulation between *fur* and *omlA*. Expression of *omlA* was constitutive regardless of growth phase, oxygen tension, iron concentration, pH, and temperature. OmlA contained a signal sequence typical of bacterial lipoproteins, with a cysteine as a putative cleavage and lipid attachment site. Inhibition of signal peptidase II by globomycin resulted in failure to process OmlA, thus giving strong evidence that OmlA is a lipoprotein. Cell fractionation followed by Western blot analysis indicated that all OmlA protein is localized in the outer membrane. Mature OmlA was an acidic (pI = 4.5) protein of 17.3 kDa and had close to 40% amino acid sequence identity to SmpA (small protein A) of *Escherichia coli*, *Vibrio cholerae*, and *Haemophilus influenzae*, a protein of unknown function. All *P. aeruginosa* strains tested as well as *Pseudomonas fluorescens* were found to produce OmlA. A mutant strain with impaired production of OmlA but no change in the expression of the overlapping *fur* gene was constructed. The *omlA* mutant was hypersusceptible to anionic detergents such as sodium dodecyl sulfate and deoxycholate, and it showed increased susceptibility to various antibiotics, including nalidixic acid, rifampin, novobiocin, and chloramphenicol. A structural role of OmlA in maintaining the cell envelope integrity is proposed.

The opportunistic pathogen *Pseudomonas aeruginosa* has the capacity to produce a large variety of virulence factors that play a role in the infection of injured or immunocompromised hosts (28, 56). The production of virulence factors is regulated in response to the environmental conditions, such as iron and oxygen availability (23, 33). Iron is frequently limiting for *P. aeruginosa*, which prefers an aerobic metabolism that requires iron-containing respiratory enzymes. *P. aeruginosa* has thus evolved powerful iron acquisition systems which can be activated upon iron starvation. The ferric uptake regulator (Fur) plays the central role in the control of the iron-regulated genes. Fur is an iron-responsive, DNA binding repressor which employs Fe(II) as a corepressor and binds as a dimer to a so-called Fur box in the promoter regions of iron-regulated genes (31). Roughly 30 targets of the *P. aeruginosa* Fur protein have been identified and were shown to be expressed in an iron-dependent manner in vivo (32). The *P. aeruginosa fur* gene is transcribed from two separate promoters which are 170 bp apart. While examining the region upstream of *fur*, we identified a novel gene which we designated *omlA*. Although *fur* and *omlA* possessed overlapping promoter regions, their functions appeared to be unrelated. OmlA represented a novel outer membrane lipoprotein which seemed to play a role in maintaining the cell envelope integrity. A large number of outer membrane proteins in *P. aeruginosa* have been characterized, and they appear to be highly conserved among the *Pseudomonadaceae* (for a review see reference 16). General functions include pore formation, transport of specific substrates, cell structure determination, and membrane stabilization. The porin class includes the major outer membrane protein OprF, which is a homolog of *Escherichia coli* OmpA (55); the highly homologous OprO and OprP, which are induced under phosphate limitation (45); OprC, a copper-binding channel protein (59); OprE, an anaerobically induced channel-forming protein (58); and components of multidrug-resistance efflux pumps such as OprM (formerly OprK) (35), OprJ (34), OprD (19), and OprN (21). Involved in the maintenance of the cell envelope are the very small OprI, which is 30% identical to Braun's lipoprotein of *E. coli* (9); OprH, which is associated with lipopolysaccharide and replaces outer membrane-stabilizing divalent cations (2); and OprL, a peptidoglycan-associated lipoprotein (22). Only three of the above proteins, OprI, OprM, and OprL, are lipoproteins, and the novel OmlA described here falls also in this category. The posttranslational modification and processing of prolipoproteins had been studied in vitro (50) and in vivo (42). They involve a few enzymatic modification steps, which include the transfer of a diacylglyceryl moiety to the sulfhydryl group of the prospective N-terminal cysteine, cleavage of the signal sequence by signal peptidase II, and acylation of the new amino terminus.

Outer membrane proteins are of great importance as vaccine candidates, since they are typically very immunogenic and have adjuvant activity. OprF and OprI have been successfully demonstrated as potential vaccines in mice and humans, either alone, or as OprF-OprI fusion proteins, or as carriers for foreign epitopes (14, 17, 52). Also, due to their conserved occurrence, outer membrane proteins are of considerable impor-

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tance in clinical diagnostics. In fact, the *oprI* lipoprotein gene has been used as a very small but specific DNA probe and as a reliable PCR target within RNA group I of the *Pseudomonadaceae* (39). Similarly, a PCR assay based on the simultaneous amplification of both *oprI* and *oprL* lipoprotein genes has been used to detect *P. aeruginosa* in clinical material with very high sensitivity and absolute specificity (8). The lipoprotein OmlA, as characterized in this report, constitutes another candidate for testing as a potential vaccine or drug target.

MATERIALS AND METHODS

Strains, plasmids, primers, and media. The relevant strains, plasmids, and primers used in this study are shown in Table 1. Luria broth (LB) was used for strain maintenance, and M9 medium containing 0.2% glucose was used for growth and susceptibility assays (40). Chelex-treated and dialyzed tryptic soy broth (D-TSB) containing 1% glycerol and 50 mM glutamate was used as low-iron medium and was supplemented with 50 μg of $FeCl_3$ per ml in high-iron medium (36). *P. aeruginosa* was grown at 32°C aerobically in shake flasks or microaerobically (5% oxygen) in static CampyPak jars. Antibiotics were used as follows: for *E. coli*, ampicillin (100 μg/ml), gentamicin (15 μg/ml), kanamycin (100 μg/ml), tetracycline (15 μg/ml), and globomycin (100 and 250 μg/ml); for *P. aeruginosa*, carbenicillin (750 μg/ml), gentamicin (75 μg/ml), tetracycline (150 μg/ml), and streptomycin (500 μg/ml); and for *Pseudomonas fluorescens*, streptomycin (500 μg/ml).

General genetic methods. PCRs were performed with *Taq* DNA polymerase (Bethesda Research Laboratories [BRL]) and appropriate custom-made primers (BRL) in a Perkin-Elmer Cetus thermal cycler, with 30 cycles of denaturing (1 min, 94°C), annealing (1 min, 54°C), and extending (1 min, 72°C), and the amplified DNA fragments were purified in a preparative agarose gel and subsequently cloned into pCRII-2.1 (Invitrogen). All cloned PCR fragments and the *omlA* gene were sequenced by the dideoxy chain termination method (41) with Sequenase 2.0 (United States Biochemical) and M13 primers or custom-made 18-mer oligonucleotides. Published procedures were followed for Southern blotting (47) and colony hybridization (12).

RNase protection analyses were performed with the Riboprobe system (Promega), and radiolabeled riboprobes from suitable cloned PCR fragments were generated by runoff transcription from the T7 promoter of linearized pCRII-2.1 as described in detail elsewhere (1). Complementary antisense RNA probes for *omlA* and *fur* were synthesized from a PCR fragment generated with the primers omlA-343 and omlA-(−101), which was cloned separately in both orientations behind the T7 promoter of pCRII-2.1 (pCRII-omlA-444a and pCRII-omlA-444b).

Translational fusions of *omlA* to the *lacZ* reporter gene on plasmid pPZ20 were constructed by directional cloning of appropriate PCR products as *Eco*RI-*Hin*dIII fragments into pPZ20. To generate the P_{omlA} PCR fragments, the primer omlA-156, which contains the *Hin*dIII restriction site, was used in combination with six primers located further upstream to yield the DNA fragments as mentioned in the text. Similarly, translational fusions of *fur* to *lacZ* were constructed by transferring previously cloned PCR products as *Eco*RI-*Pst*I fragments into plasmid pPZ30. To produce the P_{fur} PCR fragments, the primer omlA-(−35), which contains the *Pst*I restriction site, was used together with six different primers upstream of *fur*.

Overexpression and labeling of OmlA. The *omlA* coding sequence from the ATG start codon to 7 bp downstream of the TGA stop codon was amplified from chromosomal PAO1 DNA with the primers omlA-139 and omlA-676 (Table 1). The resulting 538-bp fragment was gel purified, cloned into pCRII-2.1, sequenced, and transferred as an *Nde*I-*Bam*HI fragment into the T7 expression vector pET23a, yielding pET-*omlA*. For induction and labeling experiments, *E. coli* BL21(DE3)/pLysE containing pET-*omlA* or the pET23a control vector was grown in M9 minimal medium at 37°C and induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at an optical density at 600 nm (OD_{600}) of 0.4. Rifampin (200 μg/ml) was added to 1-ml culture aliquots 30 min postinduction, and incubation was continued for 20 min. A mixture of ^{14}C-labeled amino acids (5 μCi) was added, and the cultures were shaken for 1 h. The cells were harvested, lysed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (40), and analyzed by SDS-PAGE followed by autoradiography to detect radiolabeled proteins. The above protocol was also used to monitor the processing of OmlA, with the addition of globomycin in methanol (100 and 250 μg/ml) together with the rifampin.

Generation of polyclonal anti-OmlA antibodies and Western blot analysis. A 472-bp *Nde*I/*Bam*HI fragment containing the *omlA* gene minus the first 66 bp of the coding sequence was generated by PCR with the primers omlA-205 and omlA-676 (Table 1). The DNA fragment was cloned into pCRII-2.1 and then transferred as an *Eco*RI fragment into pGEX-2T, yielding pGEX-*omlA*. The correct DNA sequence, orientation, and in-frame fusion of *gst* to *omlA* in pGEX-*omlA* was verified by sequencing. The *E. coli fur* null mutant QC1732 was transformed with pGEX-*omlA*, and 1 liter of culture was grown in LB at 37°C. The production of glutathione *S*-transferase (GST)–OmlA fusion protein was induced with 1 mM IPTG during log phase (OD_{600} = 0.4). The cells were harvested 4 h later (OD_{600} = 2.5) and resuspended in 30 ml of 50 mM Tris · HCl (pH 8.5)–150 mM NaCl. Lysis of the cells was achieved by freezing and thawing followed by sonication (six bursts of 30 s each on ice-NaCl). Cell debris were removed by centrifugation (10,000 × *g*, 10 min), and 20 ml of the soluble fraction was mixed with 2 ml of glutathione-Sepharose 4B (Pharmacia), gently agitated for 30 min at 25°C, and poured into a 5-ml spin column. After three washes of the matrix with 10 ml of phosphate-buffered saline, the bound GST-OmlA protein was specifically eluted with 4 ml of 10 mM glutathione–50 mM Tris · HCl (pH 8.0). A portion of this affinity-purified GST-OmlA fraction (2 ml, 6 mg) was mixed with 2 ml of 2× SDS sample buffer and loaded onto a 10-cm preparative 11% polyacrylamide–SDS tube gel (Bio-Rad) topped with 1.5 cm of 4% stacking gel. The gel was run at 23 ml/h at 4°C, and fractions of 2 ml were collected and subsequently analyzed for proteins on 15% minigels. Fractions containing purified GST-OmlA were pooled, and 100-μg aliquots were used without further processing for the immunization of two female New Zealand White rabbits. The antigen was administered in complete Freund's adjuvant in three weekly intervals and in incomplete Freund's adjuvant for two monthly boosters. Serum samples prepared 7 days after the last booster were used for the subsequent immunologic detection of OmlA, and preimmunization serum served as a control. Whole-cell extracts or overproduced soluble proteins were separated by SDS-PAGE on 15% acrylamide gels and blotted onto BA S-85 nitrocellulose (Schleicher & Schuell). The membranes were probed with 2,000-fold-diluted anti-OmlA serum and developed by using the Western-Light chemiluminescence detection system (Tropix Inc.). The anti-OmlA serum reacted specifically to *P. aeruginosa* OmlA that had been overproduced in *E. coli*, and preimmunization serum did not react with *P. aeruginosa* whole-cell extracts (data not shown).

Cell fractionation procedure. *P. aeruginosa* cells were fractionated following a published protocol (27) with modifications. In brief, *P. aeruginosa* was grown for 6 h in 30 ml of LB and the cells were collected by centrifugation (10,000 × *g*, 10 min), washed with 5 ml of ice-cold 20% sucrose and resuspended in 4.5 ml ice-cold 20% sucrose. The following ice-chilled solutions were slowly added: 2.25 ml of 2 M sucrose, 2.5 ml of 0.1 M Tris · HCl (pH 7.8), 0.2 ml of 25 mM Na_3EDTA (pH 8), and 0.45 ml of 0.5% lysozyme. The mixture was then incubated for 1 h at 30°C without agitation. Spheroplasts were removed by centrifugation (17,000 × *g*, 15 min), and the outer membranes were separated from the periplasmic fraction by ultracentrifugation (SW41, 30,000 rpm, 1 h). The spheroplasts were lysed osmotically in 4 volumes of 5 mM $MgCl_2$ and fractionated into intracellular proteins and inner membranes by centrifugation (20,000 × *g*, 20 min). Crude inner and outer membrane fractions were resuspended in water, while the periplasmic and intracellular proteins as well as culture supernatants were concentrated by 80% saturated ammonium sulfate and dissolved in 50 mM Tris · HCl (pH 7.5) before subsequent SDS-PAGE and Western blot analysis.

Disruption of the *omlA* gene. The PAO1 *omlA*::Tc mutant 6B, which produces extremely small amounts of OmlA protein, was constructed as follows. A 370-bp fragment comprising the 5′ portion of the *omlA* coding sequence from the ATG start codon was generated with the primers omlA-139 and omlA-508 (Table 1), cloned into pCRII-2.1, sequenced, and transferred as an *Eco*RI fragment into pSUP203 linearized with *Eco*RI. The resulting plasmid, pSUP-omlA-6B, was mobilized into *P. aeruginosa* PAO1 in a triparental mating by using *E. coli* HB101/pRK2013 as the helper strain (11, 46), and tetracycline-resistant transconjugants were isolated.

The PAO1 *omlA*::Tc mutant 3A, which lacks a functional *omlA* gene, was generated similarly, using a 291-bp PCR fragment amplified with the primers omlA-205 and omlA-495 (Table 1). A stop codon was introduced into primer omlA-495, so that the subsequent PAO1 *omlA*::Tc 3A transconjugants harbor a mutated version of the *omlA* gene with a stop codon 171 nucleotides earlier than wild-type *omlA* and thus express an OmlA protein lacking the 57 carboxy-terminal amino acids. The mutations were confirmed by Southern blotting and PCR analysis.

Nucleotide sequence accession numbers. The DNA sequence of the 2.3-kb *fur-omlA*-ORF1-ORF2 region has been deposited in GenBank under accession no. AF050676; the *omlA* gene from *P. fluorescens* has been deposited under accession no. AF050677.

RESULTS

***fur-omlA* locus and DNA sequence of the *omlA* gene.** The *fur* promoter region (36) was used in Southern blots to probe chromosomal PAO1 DNA cut with various enzymes, and a 3.6-kb *Pst*I fragment containing the region upstream of *fur* was subsequently cloned (pOML36). DNA sequence analysis of pOML36 revealed the presence of three putative open reading frames upstream of the *fur* gene (Fig. 1A). Immediately upstream and in the opposite orientation to *fur* was a 531-bp open reading frame (*omlA*), and located further upstream were two open reading frames (ORF1 and ORF2). A strong stem-loop structure between the ends of the *omlA* gene and ORF2 suggested the presence of a terminator. A portion of the DNA

TABLE 1. Strains, plasmids, and primers used in this study[a]

Strain, plasmid, or primer	Description	Reference or source
P. aeruginosa		
PAO1 (ATCC 15692)	Prototroph	18
PA103	Prototroph	24
PAK	Prototroph	30
PG201	Prototroph	15
PA1277	Prototroph	51
Ps388	Prototroph, hypotoxigenic	51
WR5	Prototroph, exotoxin A deficient	51
6B *omlA*::Tc	*omlA* mutant producing small amounts of OmlA	This study
3A *omlA*::Tc	*omlA* 3′-truncated mutant	This study
CS *fur*	Cold-sensitive *fur* mutant of PAO1	20
CSR *fur*	Spontaneous revertants of CS	This study
P. fluorescens		
ATCC 15453	Prototroph	ATCC
SBW25	Prototroph	37
E. coli		
DH5α	*hsdR recA lacZYA* φ80 *lacZ*ΔM15	BRL
HB101	*hsdS recA proA lacY*	5
QC1732	*fur* null mutant	M. McIntosh
BL21(DE3)/pLysE	High-stringency T7 expression host, *hsdS* DE3, Cm^r	Novagen
Plasmids		
pBluescript SK(+)	Amp^r *lacZ′*, cloning vector	Stratagene
pCRII 2.1	Amp^r Km^r, PCR cloning vector	Invitrogen
pGEX-2T	Amp^r, $lacI^q$, $P_{tac\text{-}gst}$ expression vector for protein fusions	Pharmacia
pSUP203	Amp^r Tc^r Cm^r mob, suicide vector	46
pVLT35	Sm^r mob, $lacI^q$, broad-host-range $P_{trp\text{-}lac}$ expression vector	7
pRK2013	Km^r conjugation helper plasmid	11
pUCP19	Amp^r P_{lacZ}, broad-host-range cloning and expression vector	43
pUCP24	Gm^r P_{lacZ}, broad-host-range cloning and expression vector	43
pPZ20, pPZ30	Amp^r, *′lacZ*-based promoter probe vector	44
pGEX-*omlA*	pGEX-2T containing a *gst-omlA* fusion under *tac* promoter control	This work
pET-*omlA*	pET23a containing *omlA* under T7 promoter control	This work
pOML15	pUCP19 containing *omlA* as a 0.8-kb *Pst*I-*Bgl*II fragment under *lac* promoter control	This work
pOML24	pUCP24 containing *omlA* as a 0.8-kb *Pst*I-*Bgl*II fragment	This work
pOML36	pUCP19 containing a 3.6-kb *Pst*I fragment of the *omlA* region	This work
pCRII-omlA-444a and pCRII-omlA-444b	pCRII containing the 444-bp *omlA-fur* promoters in *omlA* sense (a) or *fur* sense (b) orientation relative to the T7 promoter	This work
pPZ-P_{omlA}	Series of pPZ20 containing various *omlA* promoter fragments	This work
pPZ-P_{fur}	Series of pPZ30 containing various *fur* promoter fragments	This work
pPZ-PF-P_{omlA}	pPZ20 containing a 399-bp fragment of the *P. fluorescens omlA* promoter fused to *lacZ*	This work
pSUP-*omlA* 6B	pPSUP203 containing a 370-bp 5′ *omlA* fragment	This work
pSUP-*omlA* 3A	pPSUP203 containing a 291-bp internal fragment of *omlA*	This work
Primers[b]		
omlA-(−101)	CCTCGCCTGCTTCCATC	
omlA-(−35)	(*Pst*I)-CGAGCATCTGCAGGATCTTG	
omlA-139	(*Nde*I)-catATGCAAAACGCCAAGCTCATGC	
omlA-156	(*Hin*dIII)-aAGCTTGGCGTTTTGCATCG	
omlA-205	(*Nde*I)-catATGTCGTTTCCTGGCGTCTATAAAATCG	
omlA-343	GGAAGGTATCGACGATGA	
omlA-495	(Stop)-tcaGAGGATCGCTTCGTCGCGGCT	
omlA-508	TGCCTTCCTTGCCGAGGATC	
omlA-676	(*Bam*HI)-ggatCCAGCCGTCATTGCGGGCT	

[a] Abbreviations: Amp^r, ampicillin resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance; Sm^r, streptomycin resistance; Cm^r, chloramphenicol resistance; Gm^r, gentamicin resistance; ATCC, American Type Culture Collection; mob, mobilizable.

[b] The primers are numbered with respect to the DNA sequence as shown in Fig. 1. Lowercase letters at the 5′ end are nonmatching nucleotides to form the indicated motif as shown underlined.

sequence of the 2.3-kb *fur-omlA*-ORF1-ORF2 region with the *omlA* transcriptional and translational elements and the *omlA* translation is shown in Fig. 1B. The hypothetical protein sequences deduced from *omlA*, ORF1, and ORF2 were found to be homologous to corresponding *E. coli* proteins encoded in a region at 59 min of the chromosome (YfjG, YfiF, and SmpA) and to *Vibrio cholerae* proteins encoded near a pathogenicity island (ORF144 protein, ORF101 protein, and SmpA). However, these homologous genes in *E. coli* and *V. cholerae* did not map adjacent to *fur* as in *P. aeruginosa* or *P. fluorescens* but

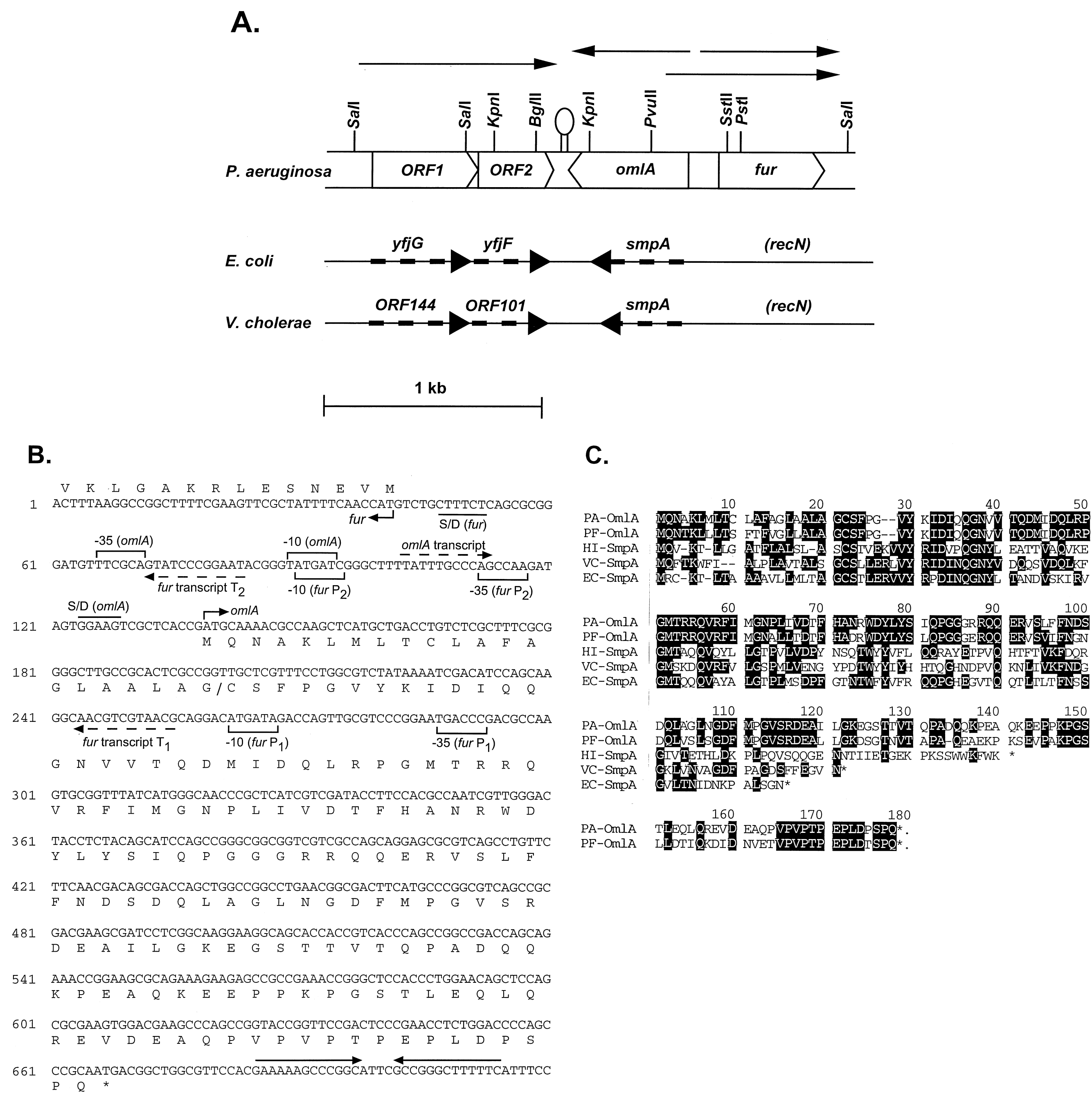

FIG. 1. (A) *P. aeruginosa* ORF1-ORF2-*omlA-fur* region. The transcripts are indicated by arrows above the corresponding genes, and an experimentally confirmed transcriptional terminator is shown between ORF2 and *omlA*. Also shown are the homologous regions of *E. coli* and *V. cholerae*, with the corresponding genes indicated by thick dashed arrows. (B) DNA sequence of the *omlA* gene. The corresponding amino acid sequence of OmlA is given below the DNA coding region. Also shown is the start of the divergent *fur* gene on the opposite DNA strand, with the amino-terminal protein sequence given above the 5′ *fur* coding sequence. Promoter elements for *omlA* and *fur* are indicated by brackets, dashed arrows represent transcriptional start sites for *omlA*, *fur* T1, and *fur* T2, and a transcriptional terminator is shown by head-to-head arrows. Also included are translational signals such as Shine-Dalgarno sequences (S/D) and the translational start sites for both *omlA* and *fur*. (C) Amino acid sequence alignment of OmlA from *P. aeruginosa* and *P. fluorescens* with SmpA from *H. influenzae* (GenBank no. 1175311), *V. cholerae* (GenBank no. U39068), and *E. coli* (GenBank no. D90888).

were located downstream of *recN*, which is located downstream of *fur* in *P. aeruginosa* (Fig. 1A). The putative 16-kDa protein encoded by ORF1 was 44% identical to *E. coli* YfjG and 49% identical to the *V. cholerae* ORF144 protein. ORF2 encoded a putative 11-kDa protein which was 43% identical to *E. coli* YfjF and 41% identical to the *V. cholerae* ORF101 protein. However, the functions of the ORF1 and ORF2 proteins and of their homologs in *E. coli* and *V. cholerae* are unknown. The deduced amino acid sequence for OmlA was homologous to a group of proteins named SmpA, for "small protein A" (Fig. 1C), although OmlA was larger and extended beyond the carboxy termini of all the SmpA proteins. *P. aeruginosa* OmlA was 73% identical to *P. fluorescens* OmlA and had also substantial identity to SmpA from *V. cholerae* (40%), *E.*

coli (39%), and *Haemophilus influenzae* (29%). An additional homolog of OmlA could be found in *Alcaligenes eutrophus*, and interestingly, this *A. eutrophus* gene was located directly upstream of *fur*, while none of the *smpA* genes map close to *fur*. However, the *A. eutrophus* homolog, which had not been annotated as a gene, may be a pseudogene, or, in the case of a sequencing error, at least needed one frameshift introduced in the reported DNA sequence to translate into a protein similar to OmlA.

Expression of *omlA* and *fur*. *omlA* gene expression was monitored at the transcriptional level by RNase protection using a riboprobe covering the *omlA* promoter. A single protected *omlA* mRNA fragment of 242 ± 3 nucleotides was detected (Fig. 2A), corresponding to a transcriptional start site at 36 ± 3 nucleotides upstream of the *omlA* start codon. Typical σ^{70}-like −35 and −10 promoter elements were located within the expected distance relative to the mapped mRNA start, as indicated in Fig. 1B. OmlA expression appeared to be constitutive regarding growth phase, iron concentration, and oxygen tension (Fig. 2A), as well as pH and temperature (data not shown). The *fur* and *omlA* genes were divergently transcribed and had overlapping promoters. In *P. aeruginosa*, transcription of *fur* was driven by two separate promoters, P1 and P2, about 170 bp apart, resulting in two *fur* transcripts, T1 and T2 (Fig. 2B). Interestingly, the distal *fur* P1 promoter and thus the start site of the T1 transcript were located well within the *omlA* coding sequence (Fig. 1B). In fact, the divergent *fur* T1 and the *omlA* transcripts overlapped by 154 bases and had the potential to form antiparallel RNA-RNA hybrids that may affect *fur* or *omlA* translation. The proximal *fur* promoter P2 driving expression of the shorter *fur* transcript T2 overlapped the *omlA* promoter so that the −10 elements of *fur* P2 and of *omlA* shared the same base pair motif on the complementary DNA strands (Fig. 1B), suggesting a potential competition for RNA polymerase binding in this region.

Expression of *omlA* and *fur* was also studied with translational fusions to the *lacZ* gene, using a series of *omlA* and *fur* promoter fragments containing increasing upstream DNA sequence as outlined in Fig. 2C. Optimal expression of *omlA* required the presence in *cis* of roughly 250 bp upstream of the −35 *omlA* promoter element. The expression of *fur-lacZ* was threefold higher when both P1 and P2 promoters were present than with P2 alone. Furthermore, *fur-lacZ* expression increased twofold and fourfold when roughly 100 and 200 bp of additional upstream DNA were included in the promoter fragment (Fig. 2C). In either case, the maximal gene expression did not require the presence of the complete upstream gene, excluding any *trans*-acting regulatory mechanism due to high levels of Fur or OmlA protein caused by multiple gene copies. Thus, it appeared rather that *cis* activation domains for optimal *fur* and *omlA* expression which were located within the coding sequence of the respective upstream gene existed (Fig. 2C).

Mutants affected in the *omlA-fur* intergenic region. Since the *omlA* and *fur* genes were highly linked to each other, we further examined the possibility whether the expression of the two genes was interdependent or whether the OmlA protein was somehow involved in Fur function and/or modification. Although *fur* was found to be essential in *P. aeruginosa*, mutant strains producing an altered Fur were obtained by screening for manganese resistance (1). Also, a conditional, cold-sensitive Fur mutant (CS) which had a Fur^- phenotype at 25°C, but not at 37°C (20), was isolated. CS was unstable and reverted spontaneously, allowing the isolation of several different revertants (CSR#0, CSR#1, and CSR#5) which appeared to be normally iron regulated and were no longer resistant to manganese. Genetic analysis of CS revealed a single base pair transition (T→C) 4 bp upstream of the *fur* start codon. The revertants had individual single base pair changes close to the original CS point mutation: CSR#0 had a C→A mutation 13 bp upstream, CSR#1 had a G→T point mutation 73 bp upstream, and CSR#5 had a C→T change 1 bp downstream of the original CS mutation site with respect to *fur* gene orientation (Fig. 3A). All these mutants were affected in the *omlA-fur* intergenic region but not in the coding regions, and the mutations had the potential to influence either transcription, mRNA stability, or translation of both genes. Analysis of *omlA* and *fur* mRNA revealed virtually identical *omlA* transcript levels in PAO1, CS, and all CSR strains (Fig. 3B, left panel). Fur transcripts T1 and T2 were detected at very similar levels in PAO1 wild-type, CS, and CSR#0; however, in CSR#1 *fur* T2 was up-regulated at least fivefold (Fig. 3B, right panel). Translation of *omlA* and *fur* was measured by translational fusions of CS and CSR *omlA* and *fur* to the *lacZ* gene (data not shown). The results were in good agreement with the direct determination of OmlA and Fur protein levels by Western blot analyses, indicating that the OmlA levels were identical (Fig. 3C, upper panel) and that the Fur levels were roughly 10-fold lower in CS, 4-fold lower in CSR#1, and 2-fold lower in CSR#1 and CSR#5 than in the PAO1 wild type (Fig. 3C, lower panel). Taken together, it appeared that the CS *fur* phenotype was caused by a translational rather than a transcriptional effect. The CS mutation between the *fur* start codon and the Shine-Dalgarno motif seemed to negatively affect translation of *fur*, resulting in a low level of Fur, which was insufficient to maintain proper iron-dependent control of Fur-regulated genes. In the revertants CSR#0 and CSR#5 the additional point mutations described above partially suppressed the translational deficiency, and the Fur levels, although still lower than in the wild type, were above the threshold concentration required for Fur-dependent gene regulation. In CSR#1, the suppressor mutation was located in the −35 region of *fur* P2 and had an up-regulating effect on *fur* T2, ultimately increasing the Fur levels. OmlA did not seem to be involved in any way in *fur* transcription or translation, and this finding was supported by the fact that the *omlA* gene on a plasmid (pOML15) did not complement the CS *fur* phenotype (data not shown).

Characterization of OmlA as an outer membrane lipoprotein. The *omlA* gene encoded an acidic protein (isoelectric point = 4.5) of 19.3 kDa which contained a 21-amino-acid-long hydrophobic signal sequence typical for bacterial lipoproteins, followed by a characteristic Cys residue at position 22, which could serve as the lipid attachment site. According to the common processing of prolipoproteins, the mature OmlA protein had an expected mass of roughly 18 kDa, which was the 17.3 kDa of the protein after cleavage of the signal sequence plus the masses of the modifying elements such as diacylglyceryl and the amino-terminal acyl group. To test whether OmlA was indeed a lipoprotein, inhibition of the lipoprotein maturation-specific signal peptidase II by globomycin was performed. Overproduction and selective labeling of OmlA in a T7 expression system in *E. coli* yielded a single radiolabeled protein migrating at roughly 24 kDa, and the addition of globomycin resulted in a slower-migrating form, presumably unmodified OmlA (Fig. 4A). Moreover, Western blot analysis of *P. aeruginosa* cell fractions clearly demonstrated that the OmlA protein was localized in the outer membrane (Fig. 4B).

A rough estimate of the number of OmlA molecules per cell was obtained by comparing Western blot signal obtained from whole-cell extracts of a known number of bacteria to the signals of serially diluted purified GST-OmlA fusion protein as a standard (Fig. 4C). Similar signal intensities were obtained with

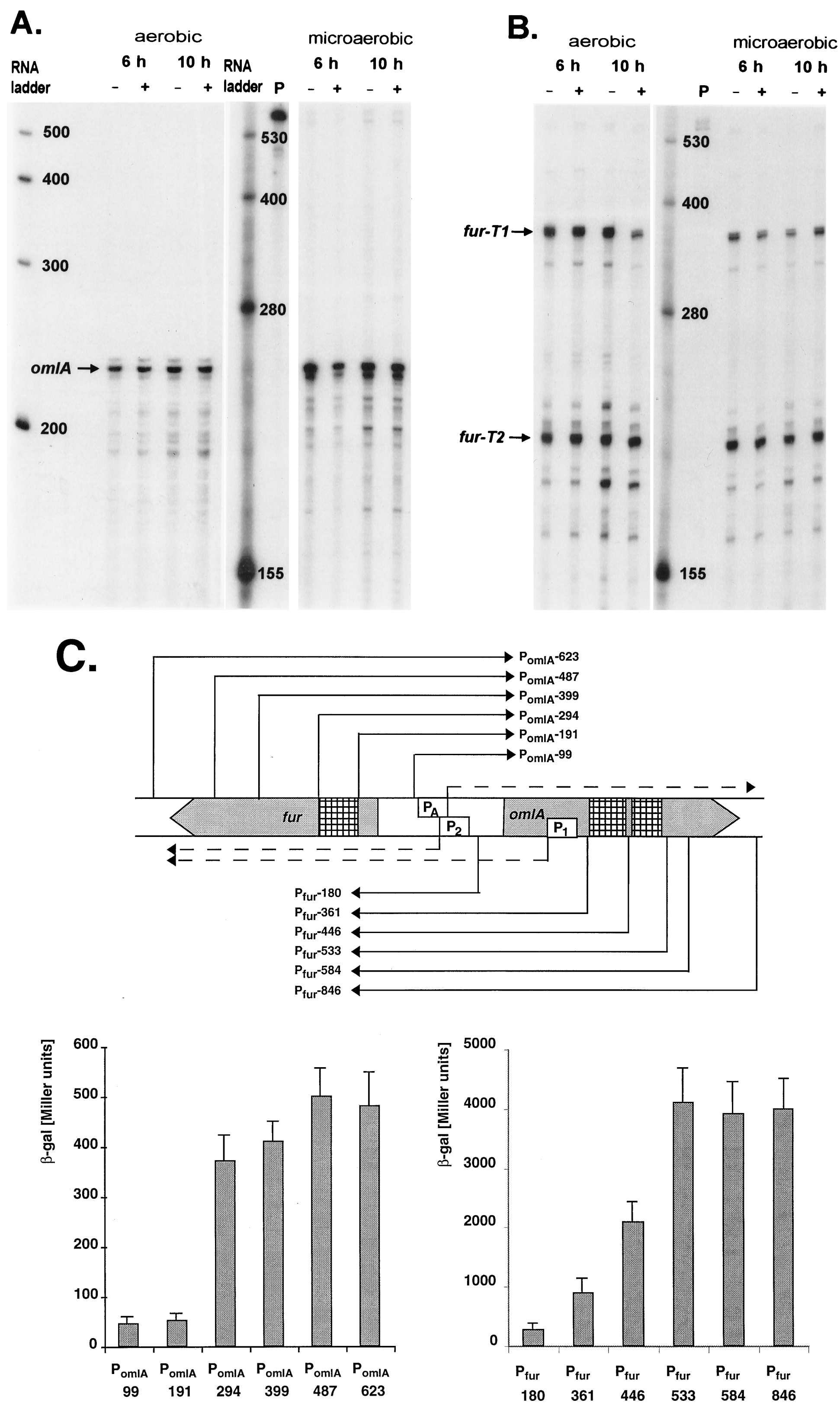

FIG. 2. (A) RNase protection analysis of *omlA* expression. A 444-nucleotide riboprobe spanning the region from 343 to −101 of the *omlA* sequence was hybridized to total RNA isolated from *P. aeruginosa* PAO1 grown for 6 or 10 h aerobically or microaerobically under low-iron (−) or high-iron (+) iron conditions. The protected *omlA* mRNA is indicated with an arrow. (B) RNase protection analysis of *fur* expression. The 444-nucleotide riboprobe covered from −101 to 343 of the *omlA* sequence, and the protected transcripts T1 and T2 are indicated by arrows. (C) Expression of *omlA* and *fur*. The map of the *fur-omlA* locus shows the *omlA* promoter P_A, the *fur* promoters P_1 and P_2, the relevant transcripts (dashed arrows), and regions for *cis* activation (hatched boxes). Promoter fragments (e.g., P_{omlA}, P_{fur}) of increasing size as indicated by arrows were translationally fused to the *lacZ* reporter gene. The β-galactosidase activities were measured in triplicate cultures grown for 4 h in D-TSB medium.

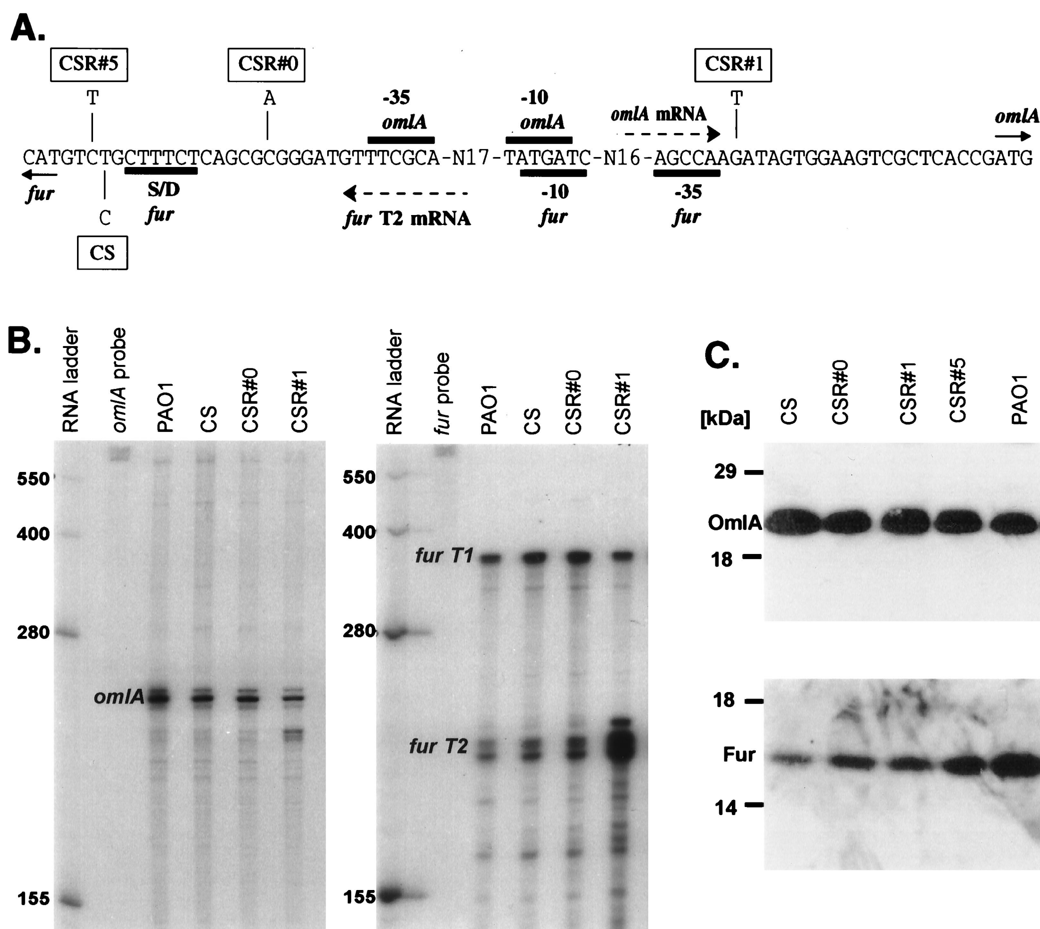

FIG. 3. (A) Locations of single base pair mutations in CS and in its revertants, CSR#0, CSR#1, and CSR#5. The relevant promoter elements, transcripts, and translational motifs within the *omlA-fur* intergenic region are indicated. S/D, Shine-Dalgarno site. (B) RNase protection of *omlA* (left) and *fur* (right) transcripts in PAO1, CS, CSR#0, and CSR#1. The RNA was isolated after 8 h of growth in high-iron D-TSB at 25°C. (C) Western blot analyses of OmlA (top) and Fur (bottom) proteins in CS, CSR#0, CSR#1, CSR#5, and wild-type PAO1. The cells were grown for 10 h in high-iron D-TSB at 25°C, and whole-cell extract samples were prepared and normalized for cell densities.

30 ng of the 43-kDa GST-OmlA fusion protein and with total proteins from 1.35×10^7 *P. aeruginosa* PAO1 cells, resulting in a calculated number of 31,000 OmlA molecules per cell.

The OmlA protein was found to be well conserved among clinical and environmental isolates of *P. aeruginosa* and was readily detectable in all strains tested, as shown for a few representative strains in Fig. 4D. Interestingly, the anti-OmlA serum did not cross-react with whole-cell extracts prepared from *P. fluorescens* (Fig. 4E). Southern blot analysis clearly demonstrated the presence of the *omlA* gene in *P. fluorescens* ATCC 15453 (data not shown), which we subsequently isolated (PF-*omlA*) to construct a *lacZ* fusion (pPZ-PF-P_{omlA}). Expression of PF-*omlA* was detectable in *P. aeruginosa* PAO1, in *P. fluorescens* ATCC 15453, and in *E. coli* DH5α and was even somewhat higher than expression of *P. aeruginosa omlA* (data not shown).

OmlA is involved in maintaining the integrity of the cell envelope. Mutants affected in the *omlA* gene were constructed in order to determine the function of the novel OmlA lipoprotein. Care had to be taken not to simultaneously alter the overlapping *fur* gene, i.e., not to disconnect the upstream *fur* P1 promoter and the activation elements which were located within the *omlA* coding sequence as demonstrated above. This was achieved by duplicating parts of the *omlA* gene and cointegration of a plasmid into the *fur-omlA* locus of PAO1 through a single crossover. The PAO1 *omlA*::Tc mutant strain 6B harbored the complete *omlA* coding sequence; however, the promoter and the ribosomal binding site were lacking (Fig. 5A). The PAO1 *omlA*::Tc mutant strain 3A possessed a truncated version of the *omlA* gene due to the introduction of an early stop codon (Fig. 5B), resulting in a hypothetical short OmlA protein that lacked 57 amino acids at the carboxy terminus. Western blot analysis of whole-cell extracts revealed extremely small amounts of OmlA in 6B, and 3A completely lacked any immunoreactive protein (Fig. 4F), suggesting that the truncated OmlA protein eventually produced in 3A was readily degraded. Complementation of 3A with the multicopy plasmid pOML24, harboring a functional *omlA* gene, restored the production of OmlA, as demonstrated by Western blot analysis (Fig. 4G). Outer membrane protein profiles from PAO1, 6B, and 3A looked virtually identical and did not discriminate the protein band corresponding to OmlA at 24 kDa on Coomassie-stained gels (Fig. 4H), although the identical samples indicated the complete loss of OmlA in 3A by Western blot analysis. Obviously, an unrelated outer membrane protein in that size range masked the OmlA protein band, or, alternatively, OmlA had disadvantageous staining properties.

The *omlA* mutant strains were tested for their susceptibility to

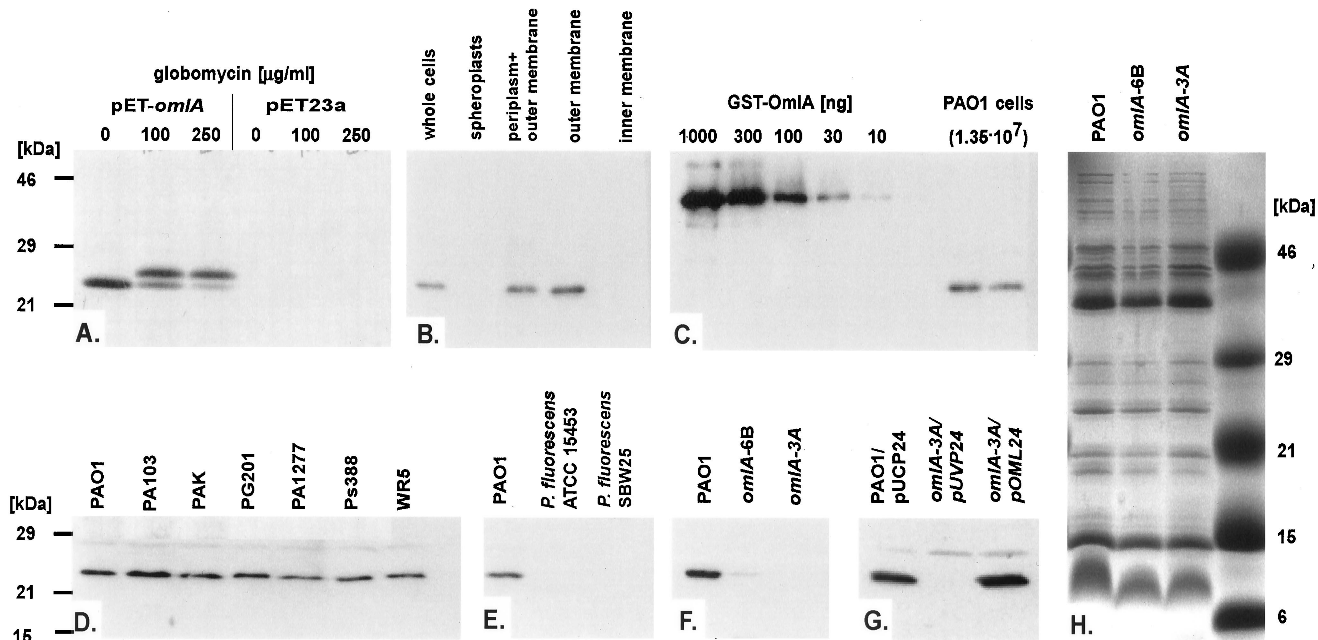

FIG. 4. (A) Autoradiography of overexpressed and radiolabeled OmlA. The *omlA* gene was expressed in a T7 system in *E. coli*/pET-*omlA* in the absence or presence of globomycin. Also shown is the pET23a vector control. (B through G) Western blot analysis of *P. aeruginosa* cell fractions, standardized GST-OmlA, and total protein from 1.35×10^7 PAO1 cells and of whole-cell extracts prepared from different *P. aeruginosa* wild-type strains, from *P. fluorescens*, from *omlA* mutants, and from a complemented *omlA* strain carrying pOML24. (H) Outer membrane protein profiles of wild-type PAO1 and *omlA* mutants on a Coomassie-stained 15% acrylamide SDS gel.

various detergents, antibiotics, and organic solvents. Mutant 6B was more susceptible and mutant 3A was hypersusceptible to anionic detergents, such as SDS and deoxycholate, but not to cationic or nonionic surface-active compounds, such as cetyltrimethylammonium bromide (CTAB), polymyxin B, Triton X-100, and Tween-20 (Table 2). Also, mutant 3A showed increased susceptibility to some antibiotics, including nalidixic acid, rifampin, novobiocin, and chloramphenicol, but not to gentamicin, cycloserine, or polymyxin B (Table 2). *P. aeruginosa* PAO1 and 3A had virtually identical growth rates and cell yields in either LB or M9 medium. Extremes in osmolarity or temperature and the presence of organic solvents such as xylene or toluene did not affect the growth characteristics of the *omlA* mutants in liquid M9 medium compared to the PAO1 wild type (data not shown). The most pronounced growth inhibition through cell lysis of 3A was found in liquid M9 medium containing SDS at concentrations of 0.05 to 0.2%. Growth and sensitivity to SDS were complemented with plasmid pOML24 supplying the *omlA* gene in *trans*; in fact, the complemented *omlA*::Tc strain 3A/pOML24 exhibited a higher resistance to SDS than wild-type PAO1 during stationary phase of growth, presumably due to a high-copy-number effect of *omlA* on pOML24 (Fig. 5C). The susceptibility of the *omlA* mutant to ionic detergents suggested a role of the OmlA protein in building the cell wall structure and, being an outer membrane protein, maintaining the integrity of the cell envelope. By using the pPZ-$P_{omlA-399}$ *omlA-lacZ* fusion in PAO1, the various compounds and conditions mentioned above were also tested for their capacity to induce up-regulation of *omlA* expression; however, such a specific stress response could not be detected (data not shown).

DISCUSSION

The *omlA* gene encoding a novel outer membrane lipoprotein has been identified, isolated, and partially characterized. It was located directly upstream of *fur* and was divergently transcribed from *fur*. The spacing of the two genes was extremely tight; in fact, the *omlA* and *fur* P2 promoters had overlapping −10 RNA polymerase binding sites on the opposite DNA strands, and the corresponding mRNA start sites were only 20 bp apart. Moreover, the upstream *fur* P1 promoter and thus the start site of the longer *fur* T1 transcript were located within the *omlA* coding sequence. As a consequence of this astonishing finding, the *omlA* transcript and the *fur* T1 transcript had an antisense overlap of at least 150 ribonucleotides. The tight spacing of two divergent promoters is a common feature in many bacteria and often occurs when two divergently transcribed genes are coregulated. Well-studied examples include the divergent *xylR* and *xylS* genes on the TOL plasmid of *Pseudomonas putida*, with a 300-bp intergenic region carrying two tandem promoters which are coregulated by XylR and IHF (25), and the divergent *tetR* and *tetA* genes of Tn*10*, which have transcriptional start sites separated by only 36 bp and are subject to coregulation by the Tet repressor (4). A highly unusual feature in bacteria, and, to our best knowledge, the first such example in *P. aeruginosa*, was our finding of truly overlapping mRNAs as in the case of *omlA* and *fur* T1. The transcriptional start site of *fur* T1 had been mapped clearly within the *omlA* coding region. Further strong evidence to support the existence of the *fur* P1 promoter was obtained by engineering a *fur* mutant strain by cointegration of a plasmid carrying the *fur* P2 promoter through single crossover into the *omlA-fur* intergenic region. In the resulting mutant the distal *fur* P1 promoter was disconnected and left the *fur* gene under control of P2 alone, without affecting the *omlA* gene at all. Interestingly, this mutant produced very small amounts of Fur and exhibited a Fur^{-} phenotype (20), which was most likely caused by the loss of the major *fur* T1 mRNA. The fact that both *omlA* and *fur*, although strongly intertwined, were func-

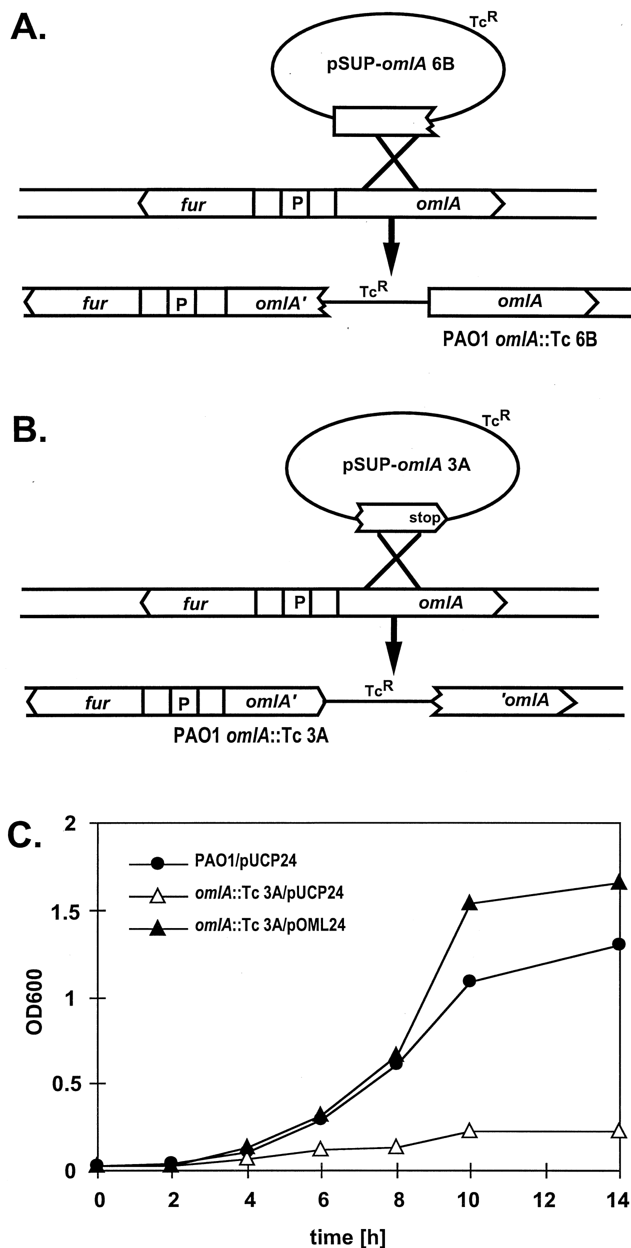

FIG. 5. (A) Construction of PAO1 *omlA*::Tc mutant 6B. The integration of the plasmid creates a promoterless *omlA* gene without affecting the divergent *fur* gene. (B) Construction of PAO1 *omlA*::Tc mutant 3A. The single crossover at *omlA* generates a truncated *omlA* gene due to an early stop codon. (C) Growth inhibition of 3A in M9 medium containing 0.1% SDS. Growth curves are shown for PAO1/pUCP24 (wild type with a control plasmid), 3A/pUCP24 (*omlA* mutant with a control plasmid), and 3A/pOML24 (*omlA* mutant genetically complemented with the *omlA* gene on a multicopy plasmid).

tional and simultaneously expressed genes raised several interesting questions regarding the bacterial transcription and translation machinery and the control thereof. The mechanism of regulation and competition for RNA polymerase binding of two overlapping divergent promoters is poorly understood. A recent study of the *E. coli fepA-fes* ferrienterochelin uptake genes which have overlapping promoters similar to *omlA* and *fur* P2 suggested that simultaneous binding of the RNA polymerase to both promoters can occur (10). Furthermore, it had

TABLE 2. Susceptibilities of wild-type PAO1 and of *omlA* mutants to various detergents and antibiotics

Compound[a]	Zone of growth inhibition (mm)[b]		
	PAO1	6B *omlA*::Tc	3A *omlA*::Tc
20% SDS	0	—[c]	13
10% Deoxycholate	0	10	13
5% CTAB	11	11	11
10% Triton X-100	14	14	14
10% Tween-20	0	0	0
0.5 M EDTA	15	15	15
2% Nalidixic acid	27	29	30
1% Rifampin	18	23	26
2% Novobiocin	19	21	24
2% Chloramphenicol	28	30	33
2% Cycloserine	10	10	10
1% Polymyxin B	22	22	22
1% Gentamicin	30	31	30

[a] The compounds were spotted (15 μl) on a sterile filter disk placed on M9 top agarose containing 10^6 cells.
[b] The zones were measured after 16 h at 37°C and are the averages for triplicate plates.
[c] Partial growth inhibition occurred in a zone of 12 mm.

to be considered that the 5′ regions of the overlapping *omlA* and *fur* T1 mRNAs had the potential to form RNA-RNA hybrids. Generally, such duplex formation may have an impact on message stability; specifically, the translational signals, including the ribosome binding site and the start codon on the *omlA* mRNA, may be masked by the overlapping *fur* T1 mRNA, which could result in a down-regulation of *omlA* translation. Such a mechanism involving RNA-RNA hybrids had been postulated to control plasmid replication (54). The production of RepA, a protein required for plasmid replication, is regulated at the posttranscriptional level by the short RNA I encoded by the *cop* gene (29). RNA I and the 5′ end of *repA* mRNA are complementary and can form a stable complex in vitro, thereby controlling RepA translation (48, 49). Alternatively, transcription and translation of *omlA* and *fur* may be strongly coupled so that any newly synthesized mRNA is quickly and repeatedly bound by ribosomes, thereby impairing the hybridization of the complementary RNAs. Following the latter scenario, the tight overlapping spacing of *omlA* and *fur* can conserve some space, in analogy to the organization of some viral genomes. It is intriguing to argue that the burying of an advantageous housekeeping gene such as *omlA* into an essential locus such as *fur* would help to conserve the *omlA* gene in the long term.

The cellular concentrations of both Fur and OmlA protein remained virtually constant over the entire growth phase and did not respond to changes in temperature, iron concentration, or oxygen tension. However, our *fur* and *omlA* expression data obtained with series of *lacZ* fusions strongly suggested the presence of upstream activation sites for both genes. The locations of these potential binding sites for a transcriptional activator were mapped within roughly 100 bp; however, further experiments are required to refine these upstream activation sites more accurately and to identify the transcriptional activators.

OmlA was demonstrated to be a lipoprotein by the inhibition of processing by globomycin. It was localized exclusively in the outer membrane according to Western blot analysis of different cell fractions. The cellular distribution of OmlA was in agreement with the nature of the OmlA signal sequence, since the amino acid residue following the prospective amino-

terminal cysteine of the mature OmlA protein was a serine. It has been shown in *E. coli* that the second amino acid residue of a lipoprotein plays a crucial role in determining its final location in the cell envelope and is therefore called the sorting signal. An aspartate residue in that position results in a cytoplasmic membrane localization, whereas other residues result in an outer membrane localization (57).

OmlA exhibited a high degree of identity to so-called SmpA (small protein A) of a few other gram-negative bacteria; however, the biological function of the SmpA homologs is unknown. Amino acid sequence analysis and motif searches revealed that SmpA proteins also harbor a lipoprotein-like signal sequence. The characterization of the highly homologous OmlA suggested that these proteins may comprise a novel family of outer membrane lipoproteins. However, the SmpA homologs were considerably smaller than OmlA because they lacked the carboxy-terminal domain of OmlA. This domain contained a helix-turn-helix motif (amino acid residues 113 to 139 of the mature OmlA protein) and the proline-rich motif PVPVPTPEPLDPSPQ (amino acid residues 141 to 155). Proline-rich proteins have been associated with aberrant migration in denaturing gels. This may explain why OmlA expressed in either *E. coli* or *P. aeruginosa* migrated at roughly 24 kDa, although its predicted size was only 18 kDa. A high proportion of prolines typically occurs immediately adjacent to α-helices, and this applied also to the OmlA protein, where the PVPVPTPEPLDPSPQ motif was located directly carboxy terminal of the helix-turn-helix. Repetitive short proline-rich sequences have been shown to play key roles in the function of *E. coli* TonB and OmpA (for a review, see reference 53). In the major outer membrane protein OmpA, which mediates F-dependent conjugation and is required for the structural integrity of the outer membrane in *E. coli*, the motif $(AP)_4$ has been proposed to act as a hinge region. In TonB, which is a key protein involved in the transport of small molecules such as iron siderophores through the cell membranes, the motif $(EP)_5X_{13}(KP)_5$ was shown to span the periplasmic space, with the $(XP)_n$ sequences acting as molecular triggers required for signal transduction across the membranes. The *omlA*::Tc mutant 3A, which produced a truncated OmlA protein lacking both the helix-turn-helix motif and the proline-rich motif, was clearly affected in its cell wall stability, suggesting that these motifs may be essential for OmlA function. Further investigations will have to focus on the topology of OmlA relative to the outer membrane. Like most outer membrane proteins, OmlA is polar overall, and hydrophobic domains are absent. Clearly, candidate motifs for the interaction of OmlA with the outer membrane are its amino terminal lipid and diacylglyceryl lipoprotein modifications and the carboxy-terminal proline-rich motif.

The precise function of OmlA remains to be elucidated. OmlA could be excluded as a porin, since the *omlA* mutants were hypersusceptible to some antibiotics whereas porin mutations are a frequent cause of high-level resistance to certain antibiotics (6, 13, 26). Similarly, OmlA was not part of a drug efflux pump because the *omlA* gene was not in a cluster with genes encoding the other efflux components typically found in all *P. aeruginosa* systems known so far, including the *mexAB-oprM* or *mexCD-oprJ* multidrug resistance operons (34). Furthermore, the antibiotics to which the *omlA* mutants were more susceptible were structurally and functionally unrelated. They belonged to different substance classes, such as quinolones, rifampins, and nitrophenyl- and dichloroacetylated compounds, and had different modes of action, such as inhibition of gyrase, transcription, and protein synthesis. Therefore, it was unlikely that the observed hypersusceptibility to these different antibiotics was due to a specific role of OmlA in binding or transport of these compounds. More plausible was a role of OmlA in maintaining the cell wall architecture, and the increased susceptibility to certain antibiotics was an indirect effect due to leakage of the cell envelope. In good agreement with this was the finding that *omlA* mutants were hypersusceptible to anionic detergents. A similar phenotype has been associated with *P. putida* mutants affected in *oprL*, which encodes the peptidoglycan-associated lipoprotein (38). Further experiments will investigate whether OmlA is associated with cell wall components such as peptidoglycan or lipopolysaccharide and whether *omlA* is somehow involved in the formation of outer membrane vesicles and/or release of periplasmic proteins into the extracellular milieu, similar to the *E. coli tol-pal* system (3). A location of OmlA at the exposed outer membrane would make it an ideal target for novel antimicrobial compounds.

ACKNOWLEDGMENTS

We thank M. Inouye for the generous gift of globomycin. The *E. coli fur* null mutant QC1732 was kindly provided by M. McIntosh (University of Missouri).

This work was supported by a grant from the National Institute of Allergy and Infectious Diseases (AI15940) to Michael L. Vasil.

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