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Identification of *rfbA*, Involved in B-Band Lipopolysaccharide Biosynthesis in *Pseudomonas aeruginosa* Serotype O5

TAPASHI DASGUPTA AND JOSEPH S. LAM*

Canadian Bacterial Diseases Network and Department of Microbiology, University of Guelph, Guelph, Ontario N1G 2W1, Canada

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Previous work from this laboratory has shown that a 26-kb insert in cosmid clone pFV100, isolated from a *Pseudomonas aeruginosa* gene library, contained genes that could restore serotype-specific B-band lipopolysaccharide (LPS) expression in rough mutant ge6. In this study, subclones from pFV100 were made to identify genes responsible for B-band LPS synthesis. Transformation of *Escherichia coli* HB101 with cosmid clone pFV100 resulted in expression of *P. aeruginosa* serotype O5 B-band LPS, indicating the presence of an *rfb* cluster in pFV100. Expression of *P. aeruginosa* LPS could not be achieved in *E. coli* HB101 transformed with any of the subclones. Complementation studies of well-characterized rough mutants of *P. aeruginosa* PAO1 deficient in B-band LPS biosynthesis were performed with the various subclones. Subclone pFV110, containing a 1.4-kb *Xba*I-*Hin*dIII insert, restored B-band LPS biosynthesis in mutant AK44 (A^+B^-; complete core). Probing chromosomal DNA from the 20 International Antigenic Typing Scheme serotypes with the 1.4-kb insert from pFV110 in Southern hybridizations revealed a positive reaction to restriction fragments in serotypes O2, O5, O16, O20, and O18. LPS of serotypes O2, O5, O16, and O20 were shown earlier to have a similar backbone structure in their O antigen. The insert in pFV110 was sequenced, and the deduced amino acid sequence was compared with sequences of protein databases. No significant homology could be detected with any sequences in the database. Open reading frame analysis identified one region, ORF303, which could encode a 33-kDa protein. Using *E. coli* maxicells for protein expression, *orf303* mediated the expression of a unique polypeptide with an apparent molecular mass of 32.5 kDa. The deficiency in the synthesis of B-band LPS biosynthesis in mutant AK44 is apparently complemented by the 33-kDa protein encoded by *orf303*. We have designated this ORF *rfbA*. This investigation is the first report on cloning and sequencing of an *rfb* gene involved specifically in O-antigen biosynthesis in *P. aeruginosa* PAO1.

Many strains of *Pseudomonas aeruginosa* coexpress two chemically and antigenically distinct forms of lipopolysaccharide (LPS), namely, A-band LPS (common antigen) and a serotype-specific B-band (O-antigenic) LPS (28, 37). A-band polysaccharide is a short-chain polymer and is composed predominantly of a repeating trisaccharide of α1→2,α1→3,α1→3-linked D-rhamnose (2). B-band LPS, on the other hand, is a structurally heterogeneous, longer-chain polymer composed of two to five different monosaccharides (25). At present, there is very little information available on the biosynthetic pathway and the regulatory genes controlling the expression of LPS in *P. aeruginosa*. Knowledge of the genes involved in LPS biosynthesis in *P. aeruginosa* becomes particularly important when one observes the changes associated with isolates from chronic infections of cystic fibrosis (CF) patients. *P. aeruginosa* isolates obtained from the sputum of CF patients earlier in the course of infection showed the presence of serotype-specific O antigen (28). However, isolates obtained during the chronic stages of infection showed three characteristics: gradual loss of serotype-specific O antigen (B-band LPS), polyagglutination in O-specific sera, and secretion of copious amounts of a mucoid exopolysaccharide called alginate (13, 18, 28, 35). Longitudinal studies of isolates of *P. aeruginosa* from a single CF patient have shown that while B-band LPS could not be detected later in the course of infection, A-band common antigen LPS synthesis was maintained and ultimately became the predominant LPS antigen on the cell surface (28). Studies by Penketh et al. (35) suggested that in chronic bronchopulmonary infection with *P. aeruginosa* in CF, the organism undergoes changes in somatic antigens in response to the CF lung environment. Alterations in the immunologically significant surface structures of *P. aeruginosa* may provide selective advantages for long-term survival of the bacterium in the CF lung; for instance, loss of O antigen in *P. aeruginosa* was shown to be associated with resistance to β-lactams (42) and aminoglycosides (23). This adaptive switch in terms of alterations on the cell surface is interesting and provides an intriguing mechanism of survival for the bacterium. The genetic basis of this switch is not clear. To obtain a better understanding of the mechanisms underlying LPS biosynthesis in *P. aeruginosa*, it is important to study the genes involved.

Lightfoot and Lam were the first to report the cloning of genes involved in the expression of A-band (29) and B-band (30) LPS of *P. aeruginosa*. A recombinant cosmid clone pFV3 complemented A-band LPS synthesis in an A-band-deficient mutant, rd7513. pFV3 also mediated A-band LPS synthesis in five of the six *P. aeruginosa* O serotypes which lack A-band LPS. Another cosmid clone, pFV100, complemented B-band LPS synthesis in mutant ge6, which lacks B-band LPS. Physical mapping of the genes involved in A-band and B-band LPS synthesis indicated that the two gene clusters are physically distinct and are separated by more than 1.9 Mbp on the *P. aeruginosa* PAO1 genome; A-band LPS genes mapped between 5.75 and 5.89 Mbp (10.5 to 13.3 min), and B-band LPS genes mapped at 1.9 Mbp (near 37 min) on the 5.9-Mbp chromosome (30).

In recent investigations, Goldberg et al. (16) and Coyne et al. (6) have reported that phosphomannomutase (the product

* Corresponding author. Phone: (519) 824-4120, ext. 3823. Fax: (519) 837-1802. Electronic mail address: jlam@micro.uoguelph.ca.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype, phenotype, or relevant properties	Reference or source
P. aeruginosa		
Serotype O5 (PAO1)	Wild type; A^+B^+	17
ge6	Mutant of PAO1; A^+B^-	30
AK44	O-antigen-deficient mutant of PAO1, complete core	26
E. coli		
CSR603	*recA1 uvrA6 phr-1 thi-1 thr-1 leuB6 lac-Y1 galK2 ara14 xyl115 mtl1 proA2 argF3 rpsL31 tsx-33 supE44 gyrA98-1* F^-	39
HB101	*supE44 hsdS20*($r_B^-m_B^-$) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1* F^- Str^r	4
DH5α	*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	GIBCO BRL
Plasmids		
pAK1900	pGEM-3Zf(+) (Promega Biotech) derivative with pRO1600 *ori* Cb^r	21, 34
pFV100	pCP13 containing cloned PAO1 genes for B-band LPS expression	30
pRK404	RK2 derivative; Tc^r Mob^+ Tra^-	9
pEX100T	Gene replacement vector; *oriT*$^+$ *sacB*$^+$ Ap^r	41
pUCGM	Source of Gm^r cassette; Ap^r Gm^r	40

of the *algC* gene), which was required for the production of mannuronic acid residues in alginate synthesis, apparently also has a role in LPS expression in *P. aeruginosa*. In their study, however, rough mutant AK1012, which is devoid of L-rhamnose and D-glucose in its outer core region (7, 26), was used for complementation studies. Thus, among other possibilities, the complementation of O-antigen biosynthesis in this mutant could be due to the involvement of the *algC* gene product in one of the earlier steps in sugar metabolism, whereby the terminal rhamnose and glucose residues in the outer core region are restored for the subsequent attachment of the O-antigen chain.

Well-defined mutants are imperative in addressing structural, biosynthetic, and molecular details of LPS in gram-negative organisms. In a recent investigation (7), we provided a comprehensive biochemical analysis of constituent sugars and an immunochemical characterization of LPS-deficient mutants derived from serotype O3 (PAC series), strain PAO1 (serotype O5; AK series), and serotype O6. The AK and PAC series of mutants have been used by several groups for the elucidation of LPS structure; however, details of the LPS-deficient or -sufficient characteristics of these *P. aeruginosa* strains were not available previously (7). The structural components elucidated for the O5- and O6-derived mutants were in agreement with the observed relative mobilities of the LPS bands in polyacrylamide gels. The serum sensitivity of these LPS-deficient mutants correlated well with the degree of roughness of the LPS. These data on the LPS core region and the A- and B-band characteristics of the mutants facilitated our present studies in identifying genes responsible for the synthesis of B-band LPS.

The present investigation reports the cloning and characterization of an *rfb* gene involved in O-antigen biosynthesis in *P. aeruginosa* serotype O5 by specific complementation of well-defined mutants that have a complete core oligosaccharide but lack the O antigen.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. All *P. aeruginosa* strains were cultured on tryptic soy broth or agar media (Difco Laboratories, Detroit, Mich.). *Escherichia coli* strains were grown in Luria broth or agar (GIBCO BRL, Toronto, Ontario, Canada). All agar media contained 1.5% Bacto Agar (Difco).

LPS analysis. Small-scale LPS preparations were obtained routinely by a proteinase K digestion method of whole bacterial cells, as described by Hitchcock and Brown (20). Samples were analyzed by discontinuous polyacrylamide gel electrophoresis (PAGE) as described by Hancock et al. (18), except that sodium dodecyl sulfate (SDS) was eliminated from the gel system; 5% stacking and 15% resolving gels were used. Silver staining for the visualization of LPS was performed by the method of Dubray and Bezard (10). Western blotting (immunoblotting) and immunodetection of the LPS were performed as described previously (7).

Recombinant DNA methods. Cosmid clone pFV100 was identified as described previously (29). Plasmids were introduced into *E. coli* DH5α by transformation (5) and into *P. aeruginosa* by electroporation (12). Plasmid DNA was isolated by standard methods as described by Sambrook et al. (38) or by the alkaline lysis method as described by Birnboim and Doly (3). DNA fragments were isolated from low-melting-point agarose gels with Geneclean (Bio 101, Inc., La Jolla, Calif.). For Southern blot analysis, DNA was transferred to Zetaprobe membranes (Bio-Rad Laboratories, Richmond, Calif.) as described in the manufacturer's instructions; DNA probes were labeled by a nonradioactive method with dUTP conjugated to digoxigenin and hybridized at 65°C for 12 h as described in the manufacturer's protocols (Boehringer-Mannheim, Laval, Quebec, Canada). Probe-target hybrids were detected with the chemiluminescent substrate AMPPD (Boehringer Mannheim) and then by exposure to a Cronex 4 X-ray film (Du Pont, Mississauga, Ontario, Canada).

Maxicell analysis of plasmid DNA. Plasmids were transformed into *E. coli* CSR603, maxicells were prepared, and expressed proteins were labeled with Trans[^{35}S]-Label (^{35}S *E. coli* hydrolysate labeling reagent containing 70% L-[^{35}S]methionine; ICN Biomedicals, Mississauga, Ontario, Canada), as described by Sancar et al. (39). Cell lysates were analyzed by SDS-PAGE, and labeled proteins were detected by autoradiography of dried gels (39). Samples were heated at 100°C for 10 min prior to electrophoresis.

Insertional inactivation of the *rfbA* gene. *P. aeruginosa rfbA* chromosomal knockout mutants were constructed with a gene replacement vector, pEX100T (41), as described by de Kievit et al. (8). An 875-bp Gm^r cassette from pUCGM (40) was cloned into the unique *Sal*I site in *rfbA*. Southern hybridization analysis with the 1.4-kb *Xba*I-*Hin*dIII (which includes the *rfbA* gene) fragment as a probe was used to confirm that allelic replacement had occurred in the *rfbA* mutants.

DNA sequencing. Both strands of DNA were sequenced completely with the Applied Biosystems (Foster City, Calif.) model 373A DNA sequencing system at the MOBIX facility, McMaster University (Hamilton, Ontario, Canada). Oligonucleotide primers were synthesized with an Applied Biosystems model 391 DNA synthesizer and purified as described in the manufacturer's instructions. Preparation of sequencing gels, buffers, and other reagents were as described in protocols provided by Applied Biosystems. All sequencing reactions were performed with the *Taq* DyeDeoxy Terminator Cycle sequencing kit from Applied Biosystems. Cycle sequencing reactions were carried out in an Ericomp (San Diego, Calif.) model TCX15 thermal cycler. At the completion of these reactions, mixtures were passed over a 1-ml Sephadex G-50 column to remove excess DyeDeoxy Terminators.

Sequence analysis. The nucleic acid sequence was assembled and analyzed with Gene Runner computer software (Hastings Software, New York, N.Y.). For sequence homology comparisons, DNA and protein sequence databases of GenBank were utilized through the NCBI BLAST network server (1, 14). Open reading frame (ORF) analysis and the predicted protein characteristics were done with the Gene Runner program.

RESULTS

Expression of *P. aeruginosa* serotype O5 O antigen in *E. coli* HB101. To investigate the possibility of the presence of an *rfb* cluster in pFV100, the cosmid clone was introduced into *E. coli* HB101 by transformation. Transformants were screened for

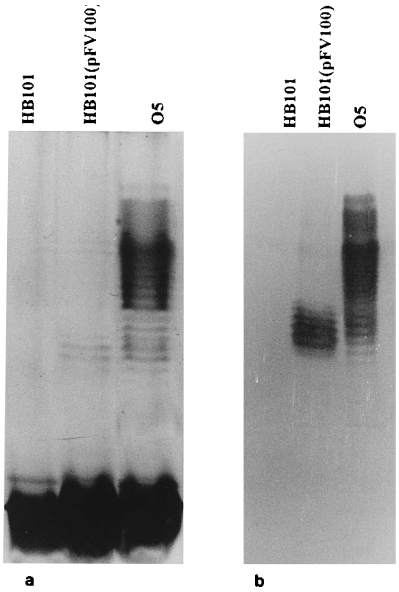

FIG. 1. Expression of *P. aeruginosa* serotype O5 O antigen in *E. coli* HB101 harboring plasmid pFV100. (a) Silver-stained polyacrylamide gel of LPS isolated from *E. coli* HB101 with and without pFV100 and of *P. aeruginosa* O5 showing the presence of a low-molecular-weight ladder pattern in *E. coli* HB101 (pFV100); (b) Western blot of LPS with serotype O5-specific MAb MF15-4 [note the expression of *P. aeruginosa* O5 O antigen in *E. coli* HB101(pFV100)].

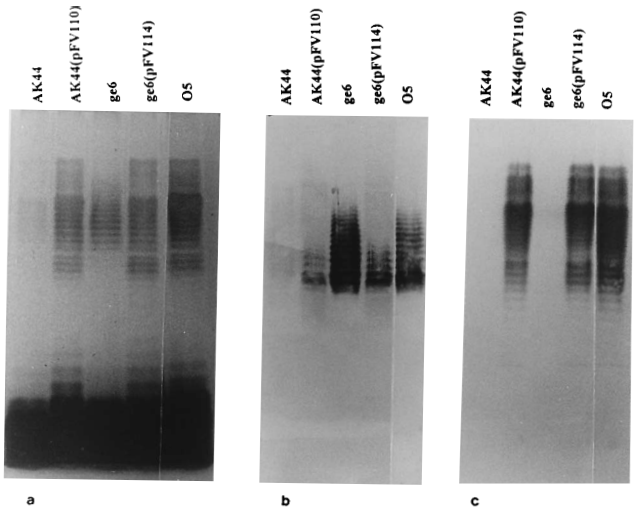

FIG. 3. (a) Silver-stained polyacrylamide gel of lipopolysaccharide from *P. aeruginosa* serotype O5 wild type and derived LPS-defective mutants. High-molecular-weight LPS bands can be seen in the mutant strains harboring plasmids. These bands could be identified as either A-band or B-band LPS by their reactions with LPS-specific MAbs. (b) Western blots of the LPS from LPS-deficient mutants, with and without plasmids, with A-band-specific MAb N1F10. Note the strong reaction of the LPS ladder in ge6 and the weak reaction with AK44 LPS, identifying the ladder pattern in these mutants as A-band LPS. (c). Western blot of the LPS with B-band-specific MAb MF15-4. A positive reaction is seen with the high-molecular-weight LPS bands of transformants AK44 (pFV110), ge6(pFV114), and wild-type O5.

the expression of *P. aeruginosa* serotype O5 O antigen by PAGE and Western blotting of the LPS with B-band-specific monoclonal antibody (MAb) MF15-4 (Fig. 1). In the silver-stained gel, ladder-like banding was observed in the *P. aeruginosa* O5 control. Faint but discernible ladderlike bands were observed in the lane with LPS from the transformant *E. coli* HB101(pFV100), whereas no high-molecular-weight LPS could be detected in the lane with HB101 LPS. Interestingly, in Western blots with MAb MF15-4, a strong reaction with short chain O-antigen polymer in *E. coli* HB101(pFV100) was observed (Fig. 1b). This indicates the presence of *P. aeruginosa* serotype O5 O-antigen epitopes in the LPS expressed in *E. coli* HB101(pFV100) (Fig. 1b). In contrast, none of the subclones caused *P. aeruginosa* O5 O-antigen expression in *E. coli* HB101.

Complementation of LPS-deficient mutants. To determine the location of LPS genes within pFV100, several subclones were used to transform *P. aeruginosa* mutants AK44 and ge6 (Fig. 2). The expression of serotype O5 O antigen in the transformants was analyzed by slide agglutination, PAGE, and silver staining analysis and by Western blotting (Fig. 3). In mutants AK44 (A^+B^-) and ge6 (A^+B^-), both of which synthesize a complete core oligosaccharide, the smallest subclones that complemented B-band LPS synthesis were pFV110 and pFV114, respectively. In transformants harboring the recombinant plasmids, their LPS banding pattern closely resembled that of the wild-type parent PAO1 (Fig. 3a). Mutant AK44 is a weak producer of A-band LPS, and hence the reaction with MAb

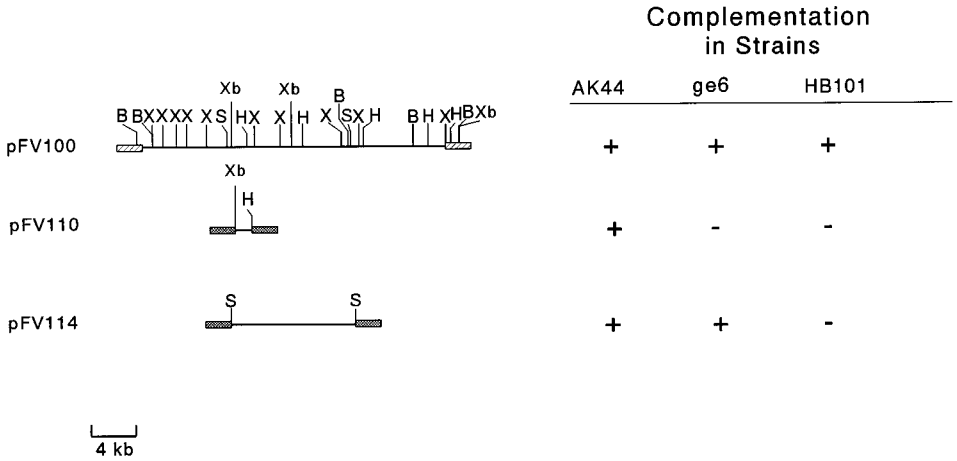

FIG. 2. Restriction map of B-band recombinant plasmid pFV100 and its subclones. Results of complementation of LPS synthesis in the *P. aeruginosa* mutants AK44 and ge6 and in *E. coli* HB101 by the various subclones are given in a table to the right of the plasmid maps. Abbreviations: B, *Bam*HI; H, *Hin*dIII; S, *Spe*I; X, *Xho*I; Xb, *Xba*I.

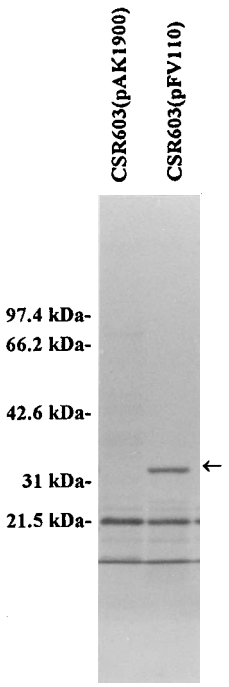

FIG. 4. Autoradiogram showing ^{35}S-labeled proteins expressed by pFV110 and corresponding plasmid vector pAK1900 in *E. coli* CSR603 by use of the maxicell system. The arrow indicates the unique 32.5-kDa polypeptide expressed in CSR603(pFV110). Molecular size markers are indicated to the left of the figure.

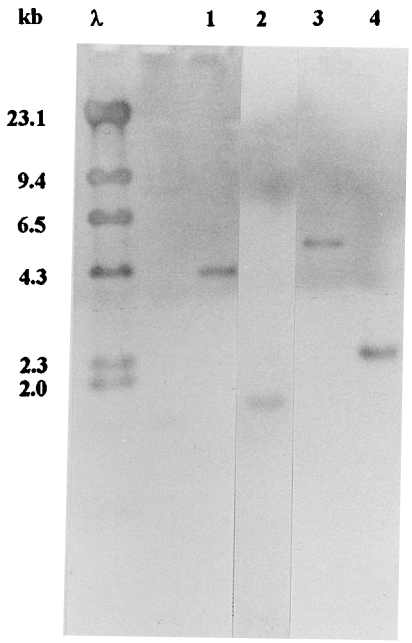

FIG. 5. Southern hybridization analysis of *rfbA* mutant M1, probed with the 1.4-kb *Xba*I-*Hin*dIII fragment. Lanes: λ, molecular mass marker of λ DNA digested with *Hin*dIII (molecular masses are indicated to the left); lane between lanes λ and 1, blank; 1 and 2, chromosomal DNA from parent *P. aeruginosa* serotype O5 digested with *Xho*I and *Xba*I-*Hin*dIII, respectively; 3 and 4, chromosomal DNA from mutant M1 digested with *Xho*I and *Xba*I-*Hin*dIII, respectively. Note the larger probe-reactive fragments in mutant M1 (lanes 3 and 4) as compared with those of the parent strain (lanes 1 and 2).

N1F10 is faint; in addition, the LPS gels and blots indicated here are a qualitative estimate rather than a quantitative evaluation of the amount of LPS elaborated by the corresponding cultures. The ladderlike band of LPS seen in mutant ge6 (A^+B^-) before complementation was identified as A-band LPS on the basis of its reactivity with MAb N1F10 (Fig. 3b), which confirms our previous results (30).

Southern hybridization analysis of serotypes O2, O16, O18, and O20. Genomic DNA from the 20 International Antigenic Typing Scheme serotypes was probed with the 1.4-kb insert from pFV110. Under high-stringency conditions, probe-reactive fragments of 23 and 4.6 kb were observed in *Bgl*II-digested and *Xho*I-digested chromosomal DNA, respectively, in serotypes O2, O5, O16, O20, AK44, and also in O18 (results not shown). No reaction with the other serotypes was discerned.

Protein expression in *E. coli* maxicells. Subclone pFV110 (1.4-kb *Xba*I-*Hin*dIII insert in vector pAK1900) was subjected to maxicell analysis in *E. coli* CSR603 to determine the number and apparent molecular weight of polypeptides involved in B-band LPS expression. A unique protein band corresponding to a polypeptide with an apparent molecular mass of 32.5 kDa was observed in maxicells harboring pFV110 (Fig. 4) but not in the control where the vector pAK1900 was transformed into *E. coli* CSR603. Another control in which the 1.4-kb fragment was cloned in the opposite orientation did not allow protein expression (data not shown).

Construction of *P. aeruginosa* chromosomal *rfbA* mutants. By use of the gene replacement system, the *rfbA* gene was rendered inactive by the insertion of the Gm^r cassette. The defective *rfbA* gene was mobilized into the parent strain O5 and integrated into the chromosome by homologous recombination. Two other markers, sucrose sensitivity (*sacB* gene) and Cb^s (i.e., loss of vector-mediated Cb^r), ensured selection of recombinants that had undergone true allelic replacement. Twenty-one Gm^r Cb^s and sucrose-sensitive colonies (putative *rfbA* mutants) were isolated. Two of these, designated M1 and M2, were chosen randomly for further analysis. By use of Southern hybridization with the 1.4-kb *Xba*I-*Hin*dIII fragment as a probe, a 5.4-kb probe-reactive fragment was detected in DNA of M1 digested with *Xho*I. In comparison, a 4.6-kb probe-reactive fragment was detected in *Xho*I-digested DNA of the control parent O5. Similarly, the probe hybridized to a 2.2-kb fragment and to a 1.4-kb fragment in *Xba*I-*Hin*dIII-digested DNA (Fig. 5). The larger probe-reactive fragments in mutant M1 are indicative of the insertion of the 875-bp Gm^r in the *rfbA* gene. In Southern analysis of mutant M2, similar results were observed (data not shown). Mutants M1 and M2 are unable to synthesize B-band LPS (Fig. 6). To further confirm that the altered phenotype in mutants M1 and M2 was due to inactivation of the *rfbA* gene, pFV110 was mobilized into these mutants. The presence of pFV110 restored B-band LPS expression in M1 and M2 (data not shown).

Sequence analysis of the 1.4-kb *Xba*I-*Hin*dIII insert from pFV110. The complete nucleotide sequence of the 1.4-kb insert was determined for both strands. The moles percent G+C of the insert in pFV110 was found to be 54.4%. One potential protein-coding region located between nucleotide positions 241 and 1152 was identified (Fig. 7). The predicted translational product of this open reading frame (ORF) is a polypeptide of 303 amino acids with a molecular mass of 33 kDa. This ORF was designated *orf303* (Fig. 7) (GenBank accession number U17293). The deduced amino acid sequence of the entire pFV110 insert was compared with peptide sequences in peptide sequence databases (SWISS-PROT release 28.0). ORF303 did not demonstrate homology to any polypeptide in the databases.

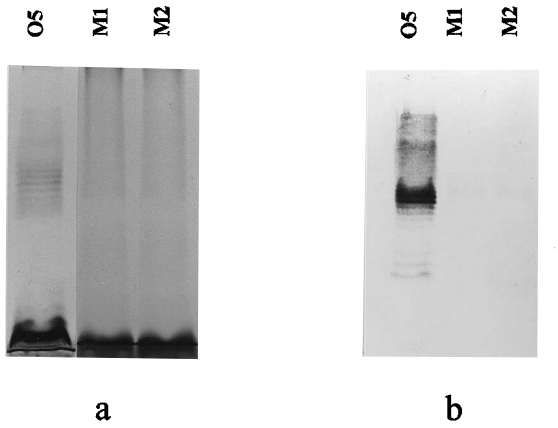

FIG. 6. Silver-stained polyacrylamide gel and Western blot of LPS from *P. aeruginosa* serotype O5 parent and two *rfbA* chromosomal mutants. (a) Silver-stained polyacrylamide gel; (b) Western blot with B-band-specific MAb MF15-4. Note the absence of B-band LPS in *rfbA* mutants M1 and M2.

Computation of hydropathy index for ORF303 product. The predicted polypeptide product from ORF303 was analyzed with Gene Runner software. The hydropathic index plot (29) indicates that the protein is predominantly hydrophobic (Fig. 8), and the protein was classified as an integral protein on the basis of the predicted membrane-spanning segments.

DISCUSSION

Earlier studies in our laboratory identified a cosmid clone, pFV100, which complemented B-band LPS synthesis in PAO1-derived B-band-deficient mutant ge6 (30). Subcloning and subsequent complementation of defined LPS-deficient mutants identified two subclones derived from pFV100 that could complement LPS deficiency (Fig. 2 and 3). The mutants used were phenotypically B^-; however, these mutants were derived by different means. Mutant AK44 (A^+B^-) was derived from PAO1 on the basis of resistance to an LPS-specific phage (26), while mutant ge6 (A^+B^-) was isolated from PAO1 by Tn*5-751* insertion mutagenesis (29). Since the LPS in mutant AK44 contains a complete core but lacks B-band LPS, complementation by subclone pFV110 (1.4-kb insert) indicated that it encodes a product required for B-band biosynthesis. The size of this insert could probably accommodate one *rfb* gene. As indicated in an earlier study (30), the loss of B-band LPS in ge6 without affecting A-band LPS indicated that the biosynthesis of these two polysaccharides is controlled independently. Subclone pFV114 which contained a 10.8-kb insert was capable of complementing the defect in ge6. In contrast, pFV110, which is a derivative of pFV114, did not restore B-band LPS synthesis in mutant ge6 (data not shown). The transposon insertion responsible for the mutation in ge6 is likely polar on downstream genes, which could explain the requirement for a larger subclone pFV114, for complementation of B-band LPS expression in this mutant.

When the 1.4-kb insert from pFV110 was used as a probe in Southern analysis, probe-reactive fragments were detected only in *P. aeruginosa* serotypes O2, O5, O16, O18, and O20. Interestingly, the O antigens of *P. aeruginosa* serotypes O2, O5, O16, and O20 share a similar sugar backbone composed of *N*-acetyl-D-fucosamine and uronic acid residues (24). The chemical structure of the O18 O antigen is not known. Thus, pFV110 likely contains an *rfb* gene that encodes an enzyme involved in the biosynthesis of a common backbone structure

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|Start orf303
    M  M  I  W  M  I  A  C  L  V  V  L  L  F  S  F  V  A  T  W
241 ATGATGATCT GGATGATCGC GTGTCTAGTT GTCTTGCTGT TTTCATTTGT CGCTACCTGG

    G  L  R  R  Y  A  L  A  T  K  L  M  D  V  P  N  A  R  S  S
301 GGGCTGCGTC GCTATGCATT AGCGACGAAA CTGATGGATG TTCCGAATGC CCGTAGCTCC

    H  S  Q  P  T  P  R  G  G  G  V  A  I  V  L  V  F  L  A  A
361 CACAGTCAAC CGACGCCTAG GGGGGGAGGT GTTGCAATCG TTCTGGTCTT CCTTGCAGCG

    L  V  W  M  L  S  A  G  S  I  S  G  G  W  G  G  A  M  L  G
421 TTGGTGTGGA TGCTGAGTGC AGGCAGTATC TCCGGCGGCT GGGGGGGGGC GATGCTGGGT

    A  G  S  G  V  A  L  L  G  F  L  D  D  H  G  H  I  A  A  R
481 GCAGGTTCTG GCGTGGCACT GTTAGGGTTC CTGGATGACC ATGGGCACAT TGCTGCGCGT

    W  R  L  L  G  H  F  S  A  A  I  W  I  L  L  W  T  G  G  F
541 TGGCGGCTGC TCGGCCATTT CTCAGCAGCG ATATGGATCT TGCTGTGGAC GGGTGGTTTC

    P  P  L  D  V  V  G  H  A  V  D  L  G  W  L  G  H  V  L  A
601 CCGCCGCTGG ATGTGGTTGG GCATGCTGTC GACTTAGGAT GGCTGGGCCA CGTATTGGCA
                              SalI

    V  F  Y  L  V  W  V  L  N  L  Y  N  F  M  D  G  I  D  G  I
661 GTTTTCTATT TGGTATGGGT GCTGAACCTT TATAACTTCA TGGATGGCAT TGATGGTATT

    A  S  V  E  A  I  G  V  C  V  G  G  A  L  I  Y  W  L  T  G
721 GCCAGTGTCG AGGCCATTGG TGTCTGTGTA GGAGGGGCCC TGATCTACTG GCTTACAGGG

    H  V  A  M  V  G  I  P  L  L  L  A  C  A  V  A  G  F  L  I
781 CATGTCGCGA TGGTTGGTAT CCCTCTGTTG CTGGCGTGCG CGGTCGCCGG CTTCCTGATC

    W  N  F  P  P  A  R  I  F  M  G  D  A  G  S  G  F  L  G  M
841 TGGAACTTCC CTCCAGCTCG AATCTTCATG GGTGATGCGG GGAGTGGTTT TCTTGGTATG

    V  I  G  A  L  A  I  Q  A  A  W  T  A  P  S  L  F  W  C  W
901 GTTATTGGTG CACTAGCTAT TCAGGCTGCA TGGACCGCCC CCTCGCTGTT CTGGTGCTGG

    L  I  L  L  G  V  F  I  V  D  A  T  Y  T  L  I  R  R  I  A
961 TTGATATTGC TGGGAGTGTT CATCGTTGAT GCAACCTATA CTCTGATCCG CCGGATCGCC

    R  G  E  K  F  Y  E  A  H  R  S  H  A  Y  Q  F  A  S  R  R
1021 AGAGGGGAGA AATTCTATGA GGCGCATCGC AGCCACGCTT ATCAGTTTGC CTCGCGTCGT

    Y  A  S  H  L  R  V  T  L  G  V  L  A  I  N  T  L  W  L  L
1081 TATGCTAGCC ATCTGCGGGT TACCTTGGGT GTTCTGGCTA TCAACACTCT TTGGTTGTTG
End of orf303|
    R  W  H  &
1141 CGTTGGCACT GA
```

FIG. 7. DNA sequence of *rfbA*. The amino acid sequence is indicated above the DNA sequence. The Gm^r cassette was inserted into the indicated unique *Sal*I site. This DNA sequence appears in the GenBank nucleotide sequence data library under the accession number U17293.

of the O antigen in these serotypes and possibly also in serotype O18.

One ORF was identified in the nucleotide sequence analysis of the insert in pFV110 (Fig. 7). The polypeptide product of this ORF is predominantly hydrophobic and is made up of 303 amino acids with a predicted size of 33 kDa. This predicted size coincided well with the size of the protein expressed in *E. coli* maxicells, which had an apparent molecular mass of 32.5 kDa. The fact that the recombinant pFV110 complements O-antigen biosynthesis in mutant AK44 and that *orf303* is the only significant protein-encoding region in the pFV110 insert indicates that we have cloned and identified an *rfb* gene from *P.*

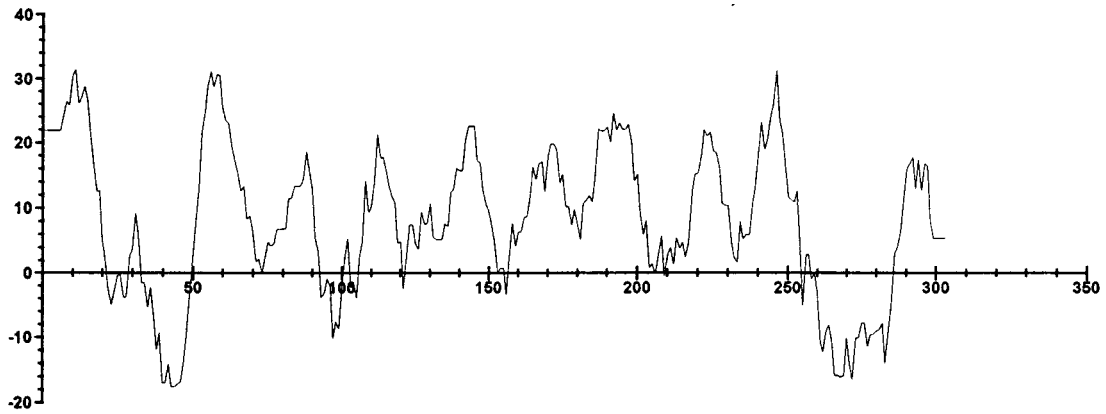

FIG. 8. Hydropathy plot of the predicted polypeptide product of *rfbA*. The numbers on the *x* axis correspond to the amino acid residue number, and those on the *y* axis correspond to the relative hydropathy index. Protein segments extending above the *x* axis (positive hydropathy index) are hydrophobic. The plot indicates that RfbA is highly hydrophobic.

aeruginosa. Since this is the first report of a gene involved in B-band LPS biosynthesis in *P. aeruginosa*, we have designated *orf303* as *rfbA*. In the homologous gene replacement experiment, insertional knockout of the *rfbA* gene affected the expression of B-band LPS. The moles percent G+C of pFV110 is 54.4%, which is well below the average moles percent G+C of *P. aeruginosa* of 65.2% (43). This observation is similar to the phenomenon observed in *Salmonella enterica* and *E. coli*, where the moles percent G+C of the *rfb* cluster is lower than that of the host organism (22, 31, 32). In a review of the evolution of *Salmonella* O-antigen variation, Reeves (36) has suggested that the differences in A+T content between the LPS gene clusters and the host chromosome could indicate that these clusters may have arisen from a series of genetic exchanges involving very dissimilar organisms. In *P. aeruginosa* serotype O5, the O antigen contains mannuronic acid residues. The *orf303*-encoded product could have a role in the conversion of an intermediate like dTDP-4-keto-6-deoxy-D-glucose to one of the residues in the O antigen of *P. aeruginosa*, probably mannuronic acid. It should be noted that in the alginate biosynthesis pathway, the *algD* gene encodes GDP-mannose dehydrogenase, which converts GDP-mannose into GDP-mannuronic acid (33). Therefore, the conversion of mannose to mannuronic acid, required for B-band LPS synthesis in serotype O5, could involve a gene which encodes TDP-mannose dehydrogenase. Alternatively, the *orf303*-encoded gene product could be involved in the biosynthesis of fucosamine, which could then be incorporated into the O antigen of *P. aeruginosa* (24). Further work is necessary to clearly define the function of this *rfbA* gene in *P. aeruginosa.*

The entire cosmid clone pFV100 could mediate *P. aeruginosa* PAO1 O-antigen expression in *E. coli* HB101 (Fig. 1), while the subclones could not. This indicates that pFV100 is sufficiently large (26-kb insert) to contain either all or a large proportion of the O-antigen *rfb* biosynthetic gene cluster. The interband spacing of the LPS expressed in *E. coli* HB101 (pFV100) is similar to that of the *P. aeruginosa* O5 LPS ladder in polyacrylamide gels (Fig. 1a). This is consistent with earlier observations from our laboratory, where LPS from the same serotype could be visualized in silver-stained polyacrylamide gels to exhibit the same interband spacing (11). However, the O antigen expressed in *E. coli* HB101(pFV100) could be a hybrid antigen containing *P. aeruginosa* serotype O5 epitopes and, partly, *E. coli* LPS polymer. A similar observation has been described by Yao and Valvano (44) in which *Shigella flexneri* O antigen was formed in *E. coli* K-12 by part host-part clone activities. Of the enteric bacteria investigated to date, the *rfb* region spans from about 8 kb in *E. coli* (19) to 20 kb in *Salmonella* spp. (22). Our results on the expression of serotype O5 O antigen in HB101 are similar to those of a previous study by Goldberg et al. (15), where *P. aeruginosa* PA103 (serotype O11) O antigen was expressed on the surface of *E. coli* HB101 harboring cosmid clone pLPS2 (containing a 26.2-kb insert).

In conclusion, we have cloned and sequenced an *rfb* gene involved in *P. aeruginosa* serotype O5 O-antigen expression. The identification of the *rfb* gene was further confirmed when knockout mutants of *rfbA* were found to be defective in B-band LPS expression. This is the first report of a specific gene that is involved directly in O-antigen expression in *P. aeruginosa*. Further sequencing and complementation studies are under way to locate other genes involved in O-antigen biosynthesis in *P. aeruginosa* serotype O5.

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ADDENDUM IN PROOF

The polypeptide product of the *rfbA* gene from *P. aeruginosa* identified in this report shows a high degree of homology (50 to 80%) with TrsF (GenBank accession no. Z47767), which is a polypeptide involved in the lipopolysaccharide outer core biosynthesis in *Yersinia enterocolitica*, serotype O:3.

REFERENCES

1. **Altschul, S. E., G. Warren, W. Miller, E. U. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Arsenault, T. L., D. W. Hughes, D. B. MacLean, W. A. Szarek, A. M. B. Kropinski, and J. S. Lam.** 1991. Structural studies of the polysaccharide portion of 'A-band' lipopolysaccharide from a mutant (AK1401) of *Pseudomonas aeruginosa* strain PAO1. Can. J. Chem. **69:**1273–1280.
3. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
4. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41:**459–496.
5. **Chung, C. T., S. L. Niemela, and R. H. Miller.** 1989. One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. Proc. Natl. Acad. Sci. USA **86:**2172–2175.

6. **Coyne, M. J., Jr., K. S. Russell, C. L. Coyle, and J. B. Goldberg.** 1994. The *Pseudomonas aeruginosa algC* gene encodes phosphoglucomutase, required for the synthesis of a complete lipopolysaccharide core. J. Bacteriol. **176:** 3500–3507.
7. **Dasgupta, T., T. R. deKievit, H. Masoud, E. Altman, J. C. Richards, I. Sadovskaya, D. Speert, and J. S. Lam.** 1994. Characterization of lipopolysaccharide-deficient mutants of *Pseudomonas aeruginosa* derived from serotypes O3, O5, and O6. Infect. Immun. **62:**809–817.
8. **de Kievit, T. R., T. Dasgupta, H. Schweizer, and J. S. Lam.** Molecular cloning and characterization of the *rfc* gene of *Pseudomonas aeruginosa* (serotype O5). Mol. Microbiol., in press.
9. **Ditta, G., T. Schmidhauser, E. Yakobson, P. Lu, X.-W. Liang, D. R. Finlay, D. Guiney, and D. R. Helinski.** 1985. Plasmids related to broad host range vector, pRK290, useful for gene cloning and for monitoring gene expression. Plasmid **13:**149–153.
10. **Dubray, G., and G. Bezard.** 1982. A highly sensitive periodic acid-silver stain for 1,2-diol groups of glycoproteins and polysaccharide in polyacrylamide gels. Anal. Biochem. **119:**325–329.
11. **Duchesne, L. G. M., J. S. Lam, L. A. MacDonald, C. Whitfield, and A. M. Kropinski.** 1988. Effect of pH and acrylamide concentration on the separation of lipopolysaccharide in polyacrylamide gels. Curr. Microbiol. **16:**191–194.
12. **Farinha, M. A., and A. M. Kropinski.** 1990. High efficiency electroporation of *Pseudomonas aeruginosa* using frozen cell suspensions. FEMS Microbiol. Lett. **70:**221–226.
13. **Fegan, M., P. Francis, A. C. Hayward, G. H. G. Davis, and J. A. Fuerst.** 1990. Phenotypic conversion of *Pseudomonas aeruginosa* in cystic fibrosis. J. Clin. Microbiol. **28:**1143–1146.
14. **Gish, W., and D. J. States.** 1993. Identification of protein coding regions by database similarity search. Nat. Genet. **3:**266–272.
15. **Goldberg, J. B., K. Hatano, G. S. Meluleni, and G. B. Pier.** 1992. Cloning and surface expression of *Pseudomonas aeruginosa* O antigen in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **89:**10716–10720.
16. **Goldberg, J. B., K. Hatano, and G. B. Pier.** 1993. Synthesis of lipopolysaccharide O side chains by *Pseudomonas aeruginosa* PAO1 requires the enzyme phosphomannomutase. J. Bacteriol. **175:**1605–1611.
17. **Hancock, R. E. W., and A. M. Carey.** 1979. Outer membrane of *Pseudomonas aeruginosa*: heat- and 2-mercaptoethanol-modifiable proteins. J. Bacteriol. **140:**902–910.
18. **Hancock, R. E. W., L. M. Mutharia, L. Chan, R. P. Darveau, D. P. Speert, and G. B. Pier.** 1983. *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis: a class of serum-sensitive, nontypable strains deficient in lipopolysaccharide O side chains. Infect. Immun. **42:**170–177.
19. **Haraguchi, G. E., U. Zähringer, B. Jann, K. Jann, R. A. Hull, and S. I. Hull.** 1991. Genetic characterization of the O4 polysaccharide gene cluster from *Escherichia coli*. Microb. Pathog. **10:**351–361.
20. **Hitchcock, P. J., and T. M. Brown.** 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. **154:**267–277.
21. **Jansons, I., G. Touchie, R. Sharp, K. Almquist, M. A. Farinha, J. S. Lam, and A. M. Kropinski.** 1994. Deletion and transposon mutagenesis and sequence analysis of the pRO1600 OriR region found in the broad-host-range plasmids of the pQF series. Plasmid **31:**265–274.
22. **Jiang, X.-M., B. Neal, F. Santiago, S. J. Lee, L. K. Romana, and P. R. Reeves.** 1991. Structure and sequence of the *rfb* (O antigen) gene cluster of *Salmonella* serovar *typhimurium* (strain LT2). Mol. Microbiol. **5:**695–713.
23. **Kadurugamuwa, J. L., J. S. Lam, and T. J. Beveridge.** 1993. Interaction of gentamicin with the A band and B band lipopolysaccharides of *Pseudomonas aeruginosa* and its possible lethal effect. Antimicrob. Agents Chemother. **37:** 715–721.
24. **Knirel, Y. A.** 1990. Polysaccharide antigens of *Pseudomonas aeruginosa*. Crit. Rev. Microbiol. **17:**273–304.
25. **Knirel, Y. A., E. V. Vinogradov, N. A. Kocharova, N. A. Paramonov, N. K. Kochetkov, B. A. Dmitriev, E. S. Stanislavsky, and B. Lányi.** 1988. The structure of O-specific polysaccharide and serological classification of *Pseudomonas aeruginosa*. Acta Microbiol. Hung. **35:**3–24.
26. **Kropinski, A. M., L. C. Chan, and F. H. Milazzo.** 1979. The extraction and analysis of lipopolysaccharide from *Pseudomonas aeruginosa* strain PAO and three rough mutants. Can. J. Microbiol. **25:**390–398.
27. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157:**105–132.
28. **Lam, M. Y. C., E. J. McGroarty, A. M. Kropinski, L. A. MacDonald, S. S. Pederson, N. Hoiby, and J. S. Lam.** 1989. The occurrence of a common lipopolysaccharide antigen in standard and clinical strains of *Pseudomonas aeruginosa*. J. Clin. Microbiol. **27:**962–967.
29. **Lightfoot, J., and J. S. Lam.** 1991. Molecular cloning of genes involved with expression of A-band lipopolysaccharide, an antigenically conserved form, in *Pseudomonas aeruginosa*. J. Bacteriol. **173:**5624–5630.
30. **Lightfoot, J., and J. S. Lam.** 1993. Chromosomal mapping, expression and synthesis of lipopolysaccharide in *Pseudomonas aeruginosa*: a role for guanosine diphospho (GDP)-D-mannose. Mol. Microbiol. **8:**771–782.
31. **Macpherson, D. F., P. A. Manning, and R. Morona.** 1994. Characterisation of the dTDP-rhamnose biosynthetic genes encoded in the *rfb* locus of *Shigella flexneri*. Mol. Microbiol. **11:**281–291.
32. **Marolda, C., and M. Valvano.** 1993. Identification, expression, and DNA sequence of the GDP-mannose biosynthesis genes encoded by the O7 *rfb* gene cluster of strain VW187 (*Escherichia coli* O7:K1). J. Bacteriol. **175:**148–158.
33. **May, T. B., D. Shinabarger, R. Maharaj, J. Kato, L. Chu, J. D. DeVault, S. Roychoudhry, N. A. Zielinski, A. Berry, R. K. Rothmel, T. K. Misra, and A. M. Chakrabarty.** 1991. Alginate synthesis by *Pseudomonas aeruginosa*: a key pathogenic factor in chronic pulmonary infection of cystic fibrosis patients. Clin. Microbiol. Rev. **4:**191–206.
34. **Olsen, R. H., G. DeBusscher, and W. R. McCombie.** 1982. Development of broad-host-range vectors and gene banks: self-cloning of *Pseudomonas aeruginosa* PAO chromosome. J. Bacteriol. **150:**60–69.
35. **Penketh, A. R. L., T. Pitt, D. Roberts, M. E. Hodson, and J. C. Batten.** 1983. The relationship of phenotype changes in *Pseudomonas aeruginosa* to clinical condition of patients with cystic fibrosis. Am. Rev. Respir. Dis. **127:**605–608.
36. **Reeves, P.** 1993. Evolution of *Salmonella* O antigen variation involved in interspecies gene transfer on a large scale. Trends Genet. **9:**17–22.
37. **Rivera, M., and E. J. McGroarty.** 1989. Analysis of a common antigen lipopolysaccharide from *Pseudomonas aeruginosa*. J. Bacteriol. **171:**2244–2248.
38. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
39. **Sancar, A., A. M. Hack, and W. D. Rupp.** 1979. Simple method for identification of plasmid-encoded proteins. J. Bacteriol. **137:**692–693.
40. **Schweizer, H. P.** 1993. Small broad-host-range gentamycin resistance gene cassettes for the site-specific insertion and deletion mutagenesis. BioTechniques **15:**831–833.
41. **Schweizer, H. P., and T. T. Hoang.** An improved system for gene replacement and *xylE* fusion analysis in *Pseudomonas aeruginosa*. Submitted for publication.
42. **Shearer, B. G., and N. J. Legakis.** 1985. *Pseudomonas aeruginosa*: evidence for the involvement of lipopolysaccharide in determining the outer membrane permeability to carbenicillin and gentamicin. J. Infect. Dis. **152:**351–355.
43. **West, S. E. H., and B. I. Iglewski.** 1988. Codon usage in *Pseudomonas aeruginosa*. Nucleic Acids Res. **16:**9323–9335.
44. **Yao, Z., and M. A. Valvano.** 1994. Genetic analysis of the O-specific lipopolysaccharide biosynthesis region (*rfb*) of *Escherichia coli* K-12 W3110: Identification of genes that confer group 6 specificity to *Shigella flexneri* serotypes Y and 4a. J. Bacteriol. **176:**4133–4143.