Synthesis of Lipopolysaccharide O Side Chains by *Pseudomonas aeruginosa* PAO1 Requires the Enzyme Phosphomannomutase

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We have cloned a lipopolysaccharide (LPS) biosynthetic gene from *Pseudomonas aeruginosa* PAO1 that complements the defect in the production and incorporation of LPS O side chains in the LPS-rough strain AK1012. This gene was characterized by pulsed-field gel electrophoresis, deletion and restriction mapping of the cloned DNA, and biochemical analysis of the protein product. The cloned DNA was found to map to the 7-to-11-min region of the *P. aeruginosa* chromosome, and the gene needed for complementation of the LPS-rough phenotype was contained on a 2.6-kb *HindIII*-*SacI* fragment. This same size restriction fragment contains the alginate gene *algC*, which encodes the enzyme phosphomannomutase (PMM) and also maps to this region of the *P. aeruginosa* chromosome. The LPS-rough strain AK1012 was deficient in PMM activity, and this activity was restored to parental levels when the cloned gene was transferred to strain AK1012. In addition, the cloned gene could complement the PMM deficiency in the *algC* mutant strain 8858, and the cloned *algC* gene could restore the LPS-smooth phenotype to strain AK1012. These results indicate that the gene we have cloned is equivalent to the alginate gene *algC*. We designate this gene *pmm* to emphasize that it encodes the enzyme PMM, which has been shown to be essential for alginate production, and we demonstrate that PMM activity is required for the LPS-smooth phenotype in *P. aeruginosa* PAO1.

Little is known about the biosynthetic or regulatory genes controlling the elaboration of lipopolysaccharide (LPS) by *Pseudomonas aeruginosa*. LPS is a major virulence factor for this species (7) and is the major target antigen for protective antibodies arising in response to acute infection and vaccination (6, 8, 44). However, among isolates of *P. aeruginosa* that chronically colonize the respiratory tract of patients with cystic fibrosis, the phenotype of the surface polysaccharides is different. Most of these strains appear mucoid upon in vitro culture because of elaboration of alginate or mucoid exopolysaccharide and are sensitive to the bactericidal effects of serum because of reduction or loss of synthesis of LPS O side chains (20, 34, 35). However, the genetic mechanisms responsible for this change and the interrelationship of alginate synthesis and O side chain elaboration have not been explored.

Recently, Lightfoot and Lam (30) reported cloning genes involved in the production of the D-rhamnan common polysaccharide antigen (A band) (39, 40) component of *P. aeruginosa* LPS by complementation of an A band-deficient mutant. This A band-deficient mutant was derived by ethyl methanesulfonate mutagenesis of an O antigen-deficient mutant, AK1401, originally isolated by its resistance to bacteriophage E79 (2). The cloned DNA was able to complement defects in five of six *P. aeruginosa* strains that are naturally A band deficient (30).

P. aeruginosa AK1012 is another LPS-rough mutant derived from strain PAO1 by its resistance to lysis by the O side chain-specific bacteriophage E79 (23). Chemical analysis by Jarrell and Kropinski (24) determined that the block in LPS synthesis in strain AK1012 was in the attachment of glucose residues to the galactosamine in the LPS core. In

addition, strain AK1012 does not incorporate A band polysaccharide antigen onto the LPS, and the DNA cloned by Lightfoot and Lam did not complement this defect (30). Here we report on the cloning of DNA from the parental strain PAO1 that restores the synthesis and attachment of O side chains onto the LPS core and also restores attachment of the D-rhamnan common polysaccharide antigen to this LPS.

We have characterized the gene on the cloned DNA that complements the LPS defect in strain AK1012 and determined that the defect responsible for the lack of O antigen production in this strain is a deficiency in phosphomannomutase (PMM) activity. PMM interconverts mannose 6-phosphate and mannose 1-phosphate. This enzymatic activity is also required for alginate production, and the gene encoding this enzyme has previously been designated *algC* (52). Here we designate this gene *pmm* and present results that indicate that synthesis of O side chains by *P. aeruginosa* PAO1 utilizes one of the same enzymatic steps required for alginate production, thereby suggesting a link between the biosyntheses of these two important surface polysaccharides in *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and DNA isolation. The *P. aeruginosa* and *Escherichia coli* strains, bacteriophage, and plasmids used in this study are described in Table 1. *P. aeruginosa* chromosomal DNA was isolated as previously described (18). Plasmids were isolated from *E. coli* by the method of Birnboim and Doly (3). Plasmids were isolated from *P. aeruginosa* strains as previously described (18). *P. aeruginosa* PAO1 and PA103 gene banks were constructed as previously described (18). The isolated plasmids were transferred to *E. coli* HB101 (5) or JM107 (33) by CaCl_2 transformation. Plasmids were transferred from *E. coli* to *P.*

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TABLE 1. Bacterial strains, bacteriophage, and plasmids used in this study

Strain, phage, or plasmid	Description ^a	Source or reference
<i>P. aeruginosa</i>		
PAO1	Fisher IT-7	29
AK1012	LPS-rough derivative of PAO1	29
Fisher IT-7	Control serotype strain	Brigham and Women's Hospital, Boston, Mass.
PA103	Fisher IT-2	31
8858	<i>algC</i> mutant	52
Bacteriophage E79	O side chain specific	29
<i>E. coli</i>		
HB101	Plasmid host	5
JM107	Plasmid host	33
Plasmids		
pLAFR1	Tc ^r broad-host-range plasmid	13
pLAFR3	<i>lacZ</i> in pLAFR1	49
pCP13	Km ^r in pLAFR1	9
pUCP18	Ap ^r broad-host-range plasmid	45
pRK2013	Km ^r helper plasmid	11
pAL1	<i>argH</i> gene in pLAFR1	D. Ohman, Memphis, Tenn.
pMMB66HE	Ap ^r broad-host-range plasmid	14
pNZ49	<i>algC</i> gene in pMMB66HE	52
pLPS1	<i>pmm</i> cloned in pLAFR1	This study
pLPS188	<i>pmm</i> gene in pUCP18	This study

^a Ap^r, ampicillin resistant; Km^r, kanamycin resistant; Tc^r tetracycline resistant.

aeruginosa by triparental mating as previously described (18) and by electroporation (47). Restriction endonucleases were obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.), and digestions were performed according to the manufacturer's instructions.

Selection for LPS-smooth transconjugants. Transconjugants of AK1012 containing DNA from gene banks from wild-type strain PAO1 or PA103 were initially selected on Bacto Cetrinide Agar (Difco Laboratories, Detroit, Mich.; to select for *P. aeruginosa* containing tetracycline (100 µg/ml; to select for plasmid-encoded antibiotic resistance). Serum-resistant strains were enriched for by growth in the presence of 25% fresh human serum, as a complement source, in 1% Proteose Peptone (20). Survivors were transferred to the same medium for a second round of enrichment. Isolated colonies were then grown for serotype analysis by agglutination with serotype-specific polyclonal rabbit antibodies (37) and by immunodiffusion with autoclave extracts of broth-grown bacteria (20) and rabbit antiserum raised against whole *P. aeruginosa* cells. Plasmids were isolated from those AK1012 transconjugants agglutinating in antiserum raised against the *P. aeruginosa* Fisher IT-7 LPS and producing a heat-stable precipitin line in immunodiffusion against serum raised against Fisher *P. aeruginosa* IT-7 cells, which was then transformed into *E. coli* as described above.

To confirm that the DNA fragment that converted strain AK1012 to an LPS-smooth strain was responsible for restor-

ing this phenotype, *E. coli* containing the pLAFR1 recombinant plasmid was transferred back to AK1012 by triparental mating. *P. aeruginosa* transconjugants were selected as described above and screened for elaboration of O side chains by colony blots (27) with O side chain-specific polyclonal sera. Selected individual colonies were further tested for O side chain elaboration by agglutination reactions and immunodiffusion as described above. In addition, several colonies were tested for elaboration of a smooth LPS by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of LPS preparations.

Antigens. LPS was extracted by the phenol-water procedure (50). Material in both the aqueous and phenol phases was recovered by ethanol precipitation (4 volumes of 95% ethanol added to each phase) and further purified by ultracentrifugation, enzyme digestion, and chromatography on columns of Sepharose CL4B, as previously described (36). Crude extracts were obtained by autoclaving 10⁹ CFU of *P. aeruginosa* for 20 min in saline and then removing the bacterial cells by centrifugation. Whole-cell lysates depleted of proteins by treatment with proteinase K were prepared as described by Hitchcock and Brown (22). LPS from *E. coli* J5 was purchased from List Biologics, Campbell, Calif.

Antiserum and MAb. Polyclonal rabbit antibodies to purified *P. aeruginosa* LPS were prepared as described previously (37). Monoclonal antibody (MAb) E87, which is specific to the D-rhamnan common antigen found among multiple *P. aeruginosa* strains (26, 43, 51), was provided by Teijin Pharmaceuticals, Tokyo, Japan.

SDS-PAGE and immunoblots. LPS fractions were subjected to SDS-PAGE on 12.5% polyacrylamide gels and visualized by silver staining as described elsewhere (27). Immunoblots were obtained by electroblot transfer of fractions from SDS-polyacrylamide gels to nitrocellulose membranes followed by incubation with either polyclonal rabbit antiserum (1:25 dilution) and horseradish peroxidase-conjugated anti-rabbit immunoglobulin G or MAb E87 and alkaline phosphatase-conjugated anti-mouse immunoglobulin G (4).

PFGE. *P. aeruginosa* strains were grown overnight in L broth. Bacterials were immobilized in 1.3% InCert Agarose and prepared by the method of Arbeit et al. (1). After treatment with lysis buffer, RNase, and lysozyme and excessive washing, plugs were digested with *Spe*I. Pulsed-field gel electrophoresis (PFGE) was performed in a RAGE apparatus (Stratagene, Inc., La Jolla, Calif.). Samples were run on 1% agarose gels at 10 to 15°C in 0.5× Tris borate EDTA (TBE) (1) at a 125° fixed angle. For initial separations of chromosomal DNA, samples were run for 20 h at 220 to 150 V (logarithmic ramp) with a pulse time of 3 to 160 s (exponential ramp). For separation of 250- to 300-kb fragments, samples were run for 36 h at 200 V (constant setting) with a pulse time of 15 to 25 s (linear ramp).

Southern blot analysis. After either agarose gel electrophoresis or PFGE, gels were prepared for Southern hybridization (48). The gels were blotted onto Duralon membranes with a Posi-blot apparatus (Stratagene). After blotting, the membranes were treated with UV light by using a Stratalinker (Stratagene). The blots were probed with DNA labelled with [³²P]dCTP by using kits for nick translation or random priming (Boehringer Mannheim) and hybridized as described previously (18).

Alginate assays. *P. aeruginosa* 8858 containing plasmids was grown on Bacto Cetrinide Agar with 500 µg of carbenicillin per ml. After 2 days of growth at room temperature, the contents of the plates were scraped into 5 ml of 0.9%

saline. Two milliliters of cells was removed and centrifuged at $27,000 \times g$ for 30 min. The supernatant was dialyzed to completion versus distilled water. The uronic acid concentration of the supernatant was determined by reactivity with carbazole (28), with alginic acid (Sigma Chemical Co., St. Louis, Mo.) as a standard. The concentration of protein from the original 2-ml cell suspension was determined with BCA protein assay reagent (Pierce Chemical Co., Rockford, Ill.), with bovine serum albumin as a standard, after 2 ml of 0.5 M NaOH was added and the mixture was boiled for 5 min. Alginate production is expressed as micrograms of uronic acid-reactive material per milligram of protein.

PMM assays. PMM assays were performed essentially as described by Zielinski et al. (52). *P. aeruginosa* was grown overnight at 37°C in L broth (or L broth containing 250 µg of carbenicillin per ml for plasmid-containing strains) with shaking at 250 rpm. Cultures were diluted 1/100 into fresh L broth and grown for 6 h. Cells were harvested by centrifugation at $8,000 \times g$ for 20 min and washed twice in 0.9% saline. The pellets were stored at -70°C until use. Cells were resuspended in a mixture of 100 mM MOPS (morpholinepropanesulfonic acid; pH 7.0), 1 mM dithiothreitol, and 10% glycerol and sonicated three times for 30 s each time with a Branson Sonifier at 20 power. The extracts were centrifuged at $40,000 \times g$ for 30 min, and the supernatant was used as a crude extract. Coupling enzymes (phosphoglucose isomerase, phosphomannose isomerase, and glucose 6-phosphate dehydrogenase) were added at 3 U each, as was 10 mM MgCl₂. Enzyme activity was determined by the increase in the optical density at 340 nm after the addition of 1 µmol of mannose 1-phosphate. One unit of PMM activity was defined as the amount leading to the reduction of 1 µmol of NADP to NADPH per min. Protein concentration was determined as described above. Specific activity is expressed as milliunits per milligram of protein from crude extracts.

RESULTS

Analysis of LPS from strains PAO1 and AK1012. LPS isolated from *P. aeruginosa* PAO1 and AK1012 was analyzed by SDS-PAGE and immunoblotting (Fig. 1). LPS from strain PAO1 had a ladder-like pattern in silver-stained SDS-polyacrylamide gels, whereas LPS from strain AK1012 appeared to be rough, composed solely of fast-moving core-like determinants. In immunoblots, the ladder-like LPS from PAO1 and the Fisher IT-7 strain reacted with antibody to O side chains and the common antigen-specific MAb E87, whereas LPS from strain AK1012 reacted with neither (Fig. 1B and C). The fast-moving core-like components visualized by immunoblotting of LPS from both PAO1 and Fisher IT-7 also reacted with antisera to O side chains (Fig. 1B), whereas the comparable component of LPS from strain AK1012 did not. This result indicates that this fastest-migrating fraction from LPS-smooth strains (PAO1 and Fisher IT-7) contains molecular species expressing short O side chains, whereas AK1012 does not. The core-like material from PAO1 and Fisher IT-7 does not express the common antigen epitope (Fig. 1C).

Derivation of LPS-smooth transconjugants from LPS-rough strain AK1012. We introduced a gene bank of DNA derived from strain PAO1 into strain AK1012 and, after enrichment for serum-resistant isolates, tested those isolates for the presence of a smooth LPS and the ability to react with O side chain-specific antibody in an immunoblot. Eight different isolates that agglutinated monospecifically with antibody to the PAO1 LPS O side chain (raised against the Fisher IT-7

FIG. 1. (A) Silver-stained gel containing purified LPS from *P. aeruginosa* Fisher IT-7 (lane 1), PAO1 (lane 2), AK1012 (lane 3), and AK1012(pLPS1) (lane 4) and *E. coli* J5 (lane 5). (B) Immunoblot of an identical gel with polyclonal rabbit antibody specific to *P. aeruginosa* Fisher IT-7 O side chains. (C) Immunoblot of an identical gel with the common antigen-specific MAb E87.

LPS) were identified. These isolates also produced an antigen that precipitated in immunodiffusion with antiserum to *P. aeruginosa* Fisher IT-7 but not with antisera to the other six *P. aeruginosa* Fisher immunotype LPSs. Strain AK1012 bearing only the cloning vector (pLAFR1) did not produce an antigen reactive in these assays. Plasmid DNA was isolated from the eight *P. aeruginosa* transconjugants and transformed into *E. coli* HB101. Restriction digest analysis indicated that all eight clones contained the same 18.2-kb *Eco*RI fragment; we designated this plasmid pLPS1. We confirmed that pLPS1 was of *P. aeruginosa* origin by its reactivity in Southern blots with DNA from strain PAO1 and its lack of reactivity with DNA from *E. coli* HB101.

When pLPS1 was transferred from *E. coli* back into the *P. aeruginosa* LPS-rough strain AK1012, all of the tetracycline-resistant transconjugants [AK1012(pLPS1) strains] expressed O side chains reactive with specific antibody in colony blots. All of the selected individual colonies were also found to grow in the presence of 25% fresh human serum, whereas strain AK1012 bearing only the cloning vector was readily killed in this serum concentration. Strain AK1012(pLPS1) became sensitive to lysis with phage E79, while strains AK1012 and AK1012(pLAFR1) were resistant to lysis by phage E79.

To determine whether DNA cloned from the PAO1 gene bank was common among *P. aeruginosa* strains, we transferred a gene bank of DNA from *P. aeruginosa* PA103 to AK1012 and performed the same series of selections and enrichments to obtain serum-resistant transconjugants. Plasmids isolated from these serum-resistant transconjugants

FIG. 2. Southern blot hybridization of pulsed-field gel containing DNA of *P. aeruginosa* strains digested with *SpeI*, with pLPS1 DNA as a probe. Numbers on the left are molecular sizes in kilobases. Lanes: 1, lambda concatemer; 2, PAO1; 3, AK1012; 4, PA103.

containing DNA cloned from strain PA103 showed a restriction endonuclease pattern identical to that of pLPS1. Plasmid pLPS1 hybridized to the same 18.2-kb *EcoRI* fragment from strain PA103 and from the recombinant plasmid containing DNA cloned from strain PA103. Chromosomal DNA isolated from strain AK1012 also hybridized to an *EcoRI* fragment of the same size as that found in pLPS1 (18.2 kb), indicating that there is no large insertion or deletion in this region that is responsible for the LPS-rough phenotype in AK1012.

Analysis of LPS from transconjugants. Analysis of LPS extracted and purified from strain AK1012(pLPS1) indicated that it had a typical ladder-like pattern following SDS-PAGE and silver staining (Fig. 1A) and reacted in immunoblots with antisera specific to the O side chain (Fig. 1B) and MAb E87 (Fig. 1C). The LPS derived from strain AK1012(pLAFR1) was visualized as a typical fast-migrating rough LPS identical to the parental LPS shown in Fig. 1. This LPS also did not react with antibody to O side chains or MAb E87.

Chromosomal mapping of cloned DNA in pLPS1. *SpeI* digests have been used to physically map the chromosome of *P. aeruginosa* PAO1 by PFGE (21, 38, 41, 42, 46). The chromosomes of strains PAO1 and AK1012 were digested with *SpeI*, and the resulting fragments were separated by PFGE. After Southern blotting and probing with pLPS1, hybridization to an ~312-kb fragment in these strains was observed (Fig. 2). A 304- to 310-kb *SpeI* fragment has been shown to map to the 7- to 11-min region of the *P. aeruginosa* PAO1 chromosome (38, 41, 42, 46). We electrophoresed parallel pulsed-field gels containing *P. aeruginosa* PAO1 chromosomal DNA and probed Southern blots with labelled pLPS1 and pAL1, which contains the *argH* gene and maps to the ~310-kb *SpeI* fragment in PAO1 (38). pLPS1 and pAL1 hybridized to the same-size *SpeI* fragment from PAO1. When pLPS1 was used as a probe, hybridization to an ~81-kb *SpeI* fragment in *P. aeruginosa* PA103 was observed (Fig. 2). This fragment corresponds in size to the fragment containing *algC* from strain PA103 (85 kb) (46).

TABLE 2. Specific activity of PMM in crude extracts of *P. aeruginosa* strains

Strain	Sp act (mU/mg)
PAO1.....	0.32
AK1012.....	BD ^a
AK1012(pMMB66HE).....	BD
AK1012(pNZ49).....	2.56
AK1012(pUCP18).....	BD
AK1012(pLPS188).....	0.21
8858(pMMB66HE).....	BD
8858(pNZ49).....	0.44
8858(pUCP18).....	BD
8858(pLPS188).....	3.58

^a BD, below detection limit.

Thus, the LPS gene maps to approximately 7 to 11 min on the PAO1 chromosome, specifically in the same region as the *algC* gene.

Restriction endonuclease map and deletion analysis of the LPS gene on pLPS1. To localize the gene required for the complementation of the mutation on AK1012, we subcloned fragments from the 18.2-kb *EcoRI* fragment contained in pLPS1. Complementation was determined after transfer of the subclones to strain AK1012 and testing of the resultant transconjugants for serum resistance and sensitivity to bacteriophage E79 and by immunoblots of isolated LPS after separation by SDS-PAGE. A 2.6-kb *HindIII-SacI* fragment cloned into the *HindIII-SacI* sites of pUCP18 yielded plasmid pLPS188, which was able to complement the LPS defect in strain AK1012. Deletion analysis indicated that the *XhoI* and *PstI* sites within this cloned region were important for restoring O side chain synthesis in strain AK1012. A restriction fragment of the same size (2.6-kb *HindIII-SstI*), containing *XhoI* and *PstI* sites required for activity, has also been described for the alginate gene *algC* (52).

Strain AK1012 is deficient in PMM. We hypothesized that the DNA cloned in pLPS188 is equivalent to the *algC* gene, which encodes the enzyme PMM. We tested whether AK1012 is deficient in this enzymatic activity. We found that the specific activity of PMM was 0.32 mU/mg in the wild-type parent strain PAO1 and that this activity was undetectable in strain AK1012. To confirm that the cloned gene could complement the PMM defect, we assayed AK1012 (pLPS188) and found that there was an increase in PMM activity to 0.21 mU/mg, while there were undetectable levels of PMM in the vector-containing strain AK1012(pUCP18) (Table 2).

To determine whether the *algC* gene could complement the PMM defect in AK1012, we introduced into this strain plasmid pNZ49, which contains the *algC* gene on a 1.8-kb fragment in the broad-host-range cloning vector pMMB66HE (52). We found that strain AK1012 (pMMB66HE) had an undetectable level of PMM activity, while AK1012(pNZ49) had a greatly increased level (2.56 mU/mg) (Table 2). In addition, AK1012(pNZ49) became sensitive to bacteriophage E79 and serum resistant. The LPS isolated from this strain elaborated long O side chains, as determined by SDS-PAGE and silver staining, and reacted with O side chain-specific antiserum in immunoblots, while the strain containing the cloning vector, AK1012 (pMMB66HE), remained E79 resistant and serum sensitive and had an LPS-rough phenotype. All of the AK1012 strains containing plasmids remained nonmucoid, a finding indicat-

ing that the cloned gene cannot independently effect the alginate phenotype in this genetic background. We have given the gene that complements AK1012 the designation *pmm* to indicate that its enzymatic activity is required for alginate production (52) and for O side chain synthesis.

The cloned *pmm* gene complements the *algC* defect in strain 8858. To test whether the cloned *pmm* gene could complement an *algC* mutation, we obtained an *algC* mutant strain from N. Zielinski and A. M. Chakrabarty. This strain, 8858, is a nonmucoid mutant strain derived from the alginate-stable, mucoid *P. aeruginosa* mutant strain 8830 by ethyl methanesulfonate mutagenesis (9). Strain 8858(pLPS188) had a mucoid phenotype and produced a high level (380 µg/ml of protein) of uronic acid-reactive material comparable to that produced by strain 8858(pNZ49) (564 µg/ml of protein), while the strains containing the cloning vectors, 8858(pUCP18) and 8858(pMMB66HE), produced only background levels (64 and 50 µg/ml of protein, respectively) of uronic acid-reactive material. These results indicate that the cloned *pmm* gene can complement the *algC* mutation in strain 8858. When these plasmid-containing strains were tested for any change in the LPS phenotype, we found that all remained sensitive to serum, were resistant to the LPS-smooth strain-specific bacteriophage E79, and had an LPS-rough phenotype as determined by SDS-PAGE. These results indicate that the cloned DNA has no effect on the LPS phenotype in this genetic background.

DISCUSSION

Our data indicate that PMM is an enzyme common to the biosynthesis of Fisher IT-7 LPS O side chains and alginate and suggest that there is a link between the production of O side chains and the production of alginate. Cloned DNA containing the *pmm* gene can complement the LPS-rough mutation in strain AK1012 but not the LPS-rough mutation in strain 8858 or other LPS-rough isolates obtained from patients with cystic fibrosis. We have recently reported the cloning of a distinctive fragment of DNA from *P. aeruginosa* PA103, designated pLPS2, that induces expression of O side chain antigens on LPS-rough strains of *P. aeruginosa* (16). We have found no homology between pLPS1 and pLPS2 (17). Introduction of the cloned *pmm* gene into nonmucoid strains other than 8858 does not induce alginate production. A different DNA fragment, isolated from the alginate switch region of the *P. aeruginosa* chromosome (12), that induces many nonmucoid strains to become mucoid, high-level alginate producers has been identified (15). However, neither 8858 nor AK1012 produced alginate after the transfer of this clone (17), indicating that PMM activity is essential for alginate production.

Our results identify PMM as a key biosynthetic enzyme in synthesis of O side chains from strain PAO1. This is not surprising, because the O side chain of this strain is composed of a trisaccharide repeat unit containing two molecules of 2-acetamido-3-acetamidino-2,3-dideoxy-mannuronic acid and one molecule of *N*-acetyl-D-fucosamine (25). PMM is a key precursor enzyme in the production of GDP-mannuronic acid, the substrate needed for synthesis of alginate. It is likely that GDP-mannuronic acid is also the precursor for the derivative of mannuronic acid incorporated into the O side chain of strain PAO1. The fact that strain AK1012 lacks any detectable O side chain antigen as part of the LPS or within cellular lysates (17) indicates the need for PMM activity for O side chain synthesis and not merely for incorporation of O side chains onto LPS cores.

With regard to this point, Jarrell and Kropinski (24) reported that the LPS defect in strain AK1012 was related to synthesis of an incomplete LPS core by this strain. They determined that strain AK1012 did not incorporate glucose or rhamnose into the growing core. It is therefore likely that the PMM defect in strain AK1012 causes both the production of a truncated LPS core and the loss of O side chain synthesis.

It is intriguing that strains of *P. aeruginosa* isolated from chronic infections in the lungs of patients with cystic fibrosis are usually mucoid, because of the high-level production of alginate, as well as LPS rough, while isolates from the environment and acute infections tend to be nonmucoid and produce a smooth LPS. The genetic and biochemical interrelationship of the production of O side chains and the production of alginate by *P. aeruginosa* has not been extensively explored. It is not known whether there are genes responsible for coregulating production of both polysaccharides, although genes regulating production of alginate have been described by a number of workers (10, 19, 32).

PMM is a biosynthetic enzyme common to production of alginate and of O side chains from strain PAO1; production of the latter likely reflects the presence of mannuronic acid-like sugars in this strain's O side chains. We have also shown that the *pmm* gene is present in strain PA103 (Fisher IT-2), which has an O side chain on its LPS composed of two molecules of *N*-acetylfucosamine and one molecule of glucose (25). We do not know whether PMM is essential for the biosynthesis of this or other O side chains, particularly those lacking mannuronic acid-like sugars, or whether PMM is needed for synthesis of other monosaccharides. The importance of PMM for synthesis of O antigens of other *P. aeruginosa* serotypes is being determined. In addition, the need for PMM activity to produce a complete LPS core to attach to the O side chain is being evaluated for other *P. aeruginosa* strains. The current work indicates that in some *P. aeruginosa* strains, cointegration of genes and their products for synthesis of both alginate and O side chains occurs. Further characterization of other relationships between alginate production and O side chain production could be a key component in understanding the pathogenesis of chronic *P. aeruginosa* infections.

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REFERENCES

1. Arbeit, R. D., M. Arthur, R. Dunn, C. Kim, R. K. Selander, and R. Goldstein. 1990. Resolution of recent evolutionary divergence among *Escherichia coli* from related lineages: the application of pulsed field electrophoresis to molecular epidemiology. *J. Infect. Dis.* 161:230-235.
2. Berry, D., and A. M. Kropinski. 1986. Effect of lipopolysaccharide mutation and temperature on plasmid transformation efficiency in *Pseudomonas aeruginosa*. *Can. J. Microbiol.* 32:436-438.
3. Birnboim, H. C., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* 7:1513-1525.
4. Blake, M. S., K. H. Johnston, G. J. Russel-Jones, and E. C. Gotschlich. 1984. A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots. *Anal. Biochem.* 136:175-179.
5. Boyer, H. W., and D. Roulland-Dussoix. 1969. A complementa-

- tion analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. 41:459-472.
6. Cryz, S. J., Jr., E. Furer, and R. Germanier. 1983. Passive protection against *Pseudomonas aeruginosa* infection in an experimental leukopenic mouse model. Infect. Immun. 40:659-664.
 7. Cryz, S. J., Jr., T. L. Pitt, E. Furer, and R. Germanier. 1984. Role of lipopolysaccharide in virulence of *Pseudomonas aeruginosa*. Infect. Immun. 44:508-513.
 8. Cryz, S. J., Jr., J. C. Sadoff, D. Ohman, and E. Furer. 1988. Characterization of the human immune response to a *Pseudomonas aeruginosa* O-polysaccharide-toxin A conjugate vaccine. J. Lab. Clin. Med. 111:701-707.
 9. Darzins, A., and A. M. Chakrabarty. 1984. Cloning of genes controlling alginate biosynthesis from a mucoid cystic fibrosis isolate of *Pseudomonas aeruginosa*. J. Bacteriol. 159:9-18.
 10. Deretic, V., C. D. Mohr, and D. W. Martin. 1991. Mucoid *Pseudomonas aeruginosa* in cystic fibrosis: signal transduction and histone-like elements in the regulation of bacterial virulence. Mol. Microbiol. 5:1577-1583.
 11. Figurski, D., and D. R. Helinski. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc. Natl. Acad. Sci. USA 76:1648-1652.
 12. Flynn, J. L., and D. E. Ohman. 1988. Cloning of genes from mucoid *Pseudomonas aeruginosa* which control spontaneous conversion to the alginate production phenotype. J. Bacteriol. 170:1452-1460.
 13. Friedman, A. M., S. R. Long, S. E. Brown, W. J. Buikema, and F. M. Ausubel. 1982. Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. Gene 18:289-296.
 14. Furst, J. P., W. Pansegrau, R. Frank, H. Blocker, P. Scholz, M. Bagdasarian, and E. Lanka. 1986. Molecular cloning of the plasmid RP4 primase region in a multi-host-range *tacP* expression vector. Gene 48:119-131.
 15. Goldberg, J. B., W. L. Gorman, J. L. Flynn, and D. E. Ohman. 1993. A mutation in *algN* permits trans activation of alginate production by *algT* in *Pseudomonas* species. J. Bacteriol. 175:1303-1308.
 16. 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.
 17. Goldberg, J. B., K. Hatano, and G. B. Pier. Unpublished data.
 18. Goldberg, J. B., and D. E. Ohman. 1984. Cloning and expression in *Pseudomonas aeruginosa* of a gene involved in the production of alginate. J. Bacteriol. 158:1115-1121.
 19. Goldberg, J. B., and D. E. Ohman. 1987. Construction and characterization of *Pseudomonas aeruginosa* *algB* mutants: role of *algB* in high-level production of alginate. J. Bacteriol. 169:1593-1602.
 20. 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.
 21. Hector, J. S. R., and A. R. Johnson. 1990. Determination of genome size of *Pseudomonas aeruginosa* by PFGE: analysis of restriction fragments. Nucleic Acids Res. 18:3171-3174.
 22. Hitchcock, P. J., and T. M. Brown. 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. 154:269-277.
 23. Jarrell, K., and A. M. Kropinski. 1977. The chemical composition of the lipopolysaccharide from *Pseudomonas aeruginosa* strain PAO and a spontaneously derived rough mutant. Microbios 19:103-116.
 24. Jarrell, K. F., and A. M. Kropinski. 1981. *Pseudomonas aeruginosa* bacteriophage ϕ PLS27-lipopolysaccharide interactions. J. Virol. 40:411-420.
 25. Knirel, Y. A., E. V. Vinogradov, N. A. Kocharova, N. A. Paramonov, N. K. Kochetkov, B. A. Dmitriev, E. S. Stanislavsky, and B. Lanyi. 1988. The structure of O-specific polysaccharides and serological classification of *Pseudomonas aeruginosa*. Acta Microbiol. Hung. 35:3-24.
 26. Kocharova, N. A., K. Hatano, A. S. Shashkov, Y. A. Knirel, N. K. Kochetkov, and G. B. Pier. 1989. The structure and serologic distribution of an extracellular neutral polysaccharide from *Pseudomonas aeruginosa* immunotype-3. J. Biol. Chem. 264:15569-15573.
 27. Kocharova, N. A., Y. A. Knirel, A. S. Shashkov, N. K. Kochetkov, and G. B. Pier. 1988. Structure of an extracellular, cross reactive polysaccharide from *Pseudomonas aeruginosa* immunotype 4. J. Biol. Chem. 263:11291-11295.
 28. Kosakai, M., and Z. Yosizawa. 1979. A partial modification of the carbazole method of Bitter and Muir for quantitation of hexuronic acids. Anal. Biochem. 93:295-298.
 29. Kropinski, A. M., L. C. Chan, and F. H. Milazzo. 1979. The extraction and analysis of lipopolysaccharides from *Pseudomonas aeruginosa* strain PAO, and three rough mutants. Can. J. Microbiol. 25:390-398.
 30. 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.
 31. Liu, P. V. 1973. Exotoxins of *Pseudomonas aeruginosa*. I. Factors that influence the production of exotoxin A. J. Infect. Dis. 128:506-513.
 32. May, T. B., D. Shinabarger, R. Maharaj, J. Kato, L. Chu, J. D. DeVault, S. Roychoudhury, 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.
 33. Messing, J. 1983. New M13 vectors for cloning. Methods Enzymol. 101:20-78.
 34. Ojieniyi, B., L. Baek, and N. Hoiby. 1985. Polyagglutinability due to loss of O-antigenic determinants in *Pseudomonas aeruginosa* strains isolated from cystic fibrosis patients. Acta Pathol. Microbiol. Scand. Sect. B 93:7-13.
 35. Penketh, A., T. Pitt, D. Roberts, M. E. Hodson, and J. C. Batten. 1983. The relationship of phenotype changes in *Pseudomonas aeruginosa* to the clinical condition of patients with cystic fibrosis. Am. Rev. Respir. Dis. 127:605-608.
 36. Pier, G. B., H. F. Sidberry, S. Zolyomi, and J. C. Sadoff. 1978. Isolation and characterization of a high-molecular-weight polysaccharide from the slime of *Pseudomonas aeruginosa*. Infect. Immun. 22:908-918.
 37. Pier, G. B., and D. M. Thomas. 1982. Lipopolysaccharide and high molecular weight polysaccharide serotypes of *Pseudomonas aeruginosa*. J. Infect. Dis. 145:217-223.
 38. Ratnaningsih, E., S. Dharmsthiti, V. Krishnapillai, A. Morgan, M. Sinclair, and B. W. Holloway. 1990. A combined physical and genetic map of *Pseudomonas aeruginosa* PAO. J. Gen. Microbiol. 136:2351-2357.
 39. Rivera, M., L. E. Bryan, R. E. W. Hancock, and E. J. McGroarty. 1988. Heterogeneity of lipopolysaccharide from *Pseudomonas aeruginosa*: analysis of lipopolysaccharide chain length. J. Bacteriol. 170:512-521.
 40. Rivera, M., and E. J. McGroarty. 1989. Analysis of a common-antigen lipopolysaccharide from *Pseudomonas aeruginosa*. J. Bacteriol. 171:2244-2248.
 41. Romling, U., D. Grothues, W. Bautsch, and B. Tummler. 1989. A physical genome map of *Pseudomonas aeruginosa* PAO. EMBO J. 8:4081-4089.
 42. Romling, U., and B. Tummler. 1991. The impact of two-dimensional pulsed-field gel electrophoresis techniques for the consistent and complete mapping of bacterial genomes: refined physical map of *Pseudomonas aeruginosa* PAO. Nucleic Acids Res. 19:3199-3206.
 43. Sawada, S., T. Kawamura, Y. Masuho, and K. Tomibe. 1985. A new common polysaccharide antigen of strains of *Pseudomonas aeruginosa* detected with a monoclonal antibody. J. Infect. Dis. 152:1290-1299.

44. Schaad, U. B., A. B. Lang, J. Wedgwood, A. Ruedeberg, J. U. Que, E. Furer, and S. J. Cryz. 1991. Safety and immunogenicity of *Pseudomonas aeruginosa* conjugate-A vaccine in cystic fibrosis. *Lancet* **338**:1236-1237.
45. Schweizer, H. P. 1991. *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19. *Gene* **97**:109-112.
46. Shortridge, V. D., M. L. Pato, A. I. Vasil, and M. L. Vasil. 1991. Physical mapping of virulence-associated genes in *Pseudomonas aeruginosa* by transverse alternating-field electrophoresis. *Infect. Immun.* **59**:3596-3603.
47. Smith, A. W., and B. H. Iglewski. 1989. Transformation of *Pseudomonas aeruginosa* by electroporation. *Nucleic Acids Res.* **17**:10509.
48. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**:503-511.
49. Staskawicz, B., D. Dahlbeck, N. Keen, and C. Napoli. 1987. Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. *J. Bacteriol.* **169**:5789-5794.
50. Westphal, O., and K. Jann. 1965. Bacterial lipopolysaccharides: extraction with phenol-water and further applications of the procedure. *Methods Carbohydr. Chem.* **5**:83-91.
51. Yokota, S., S. Kaya, Y. Araki, E. Ito, T. Kawamura, and S. Sawada. 1990. Occurrence of D-rhamnan as the common antigen reactive against monoclonal antibody E87 in *Pseudomonas aeruginosa* IFO 3080 and other strains. *J. Bacteriol.* **172**:6162-6164.
52. Zielinski, N., A. Chakrabarty, and A. Berry. 1991. Characterization and regulation of the *Pseudomonas aeruginosa algC* gene encoding phosphomannomutase. *J. Biol. Chem.* **266**:9754-9763.