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Role of Phosphoglucomutase of *Stenotrophomonas maltophilia* in Lipopolysaccharide Biosynthesis, Virulence, and Antibiotic Resistance

Geoffrey A. McKay,[1] Donald E. Woods,[2] Kelly L. MacDonald,[3] and Keith Poole[1]*

Department of Microbiology and Immunology, Queen's University, Kingston, Ontario, Canada K7L 3N6[1]; Department of Microbiology and Infectious Diseases, Faculty of Medicine, University of Calgary Health Sciences Centre, Calgary, Alberta, Canada T2N 4N1[2]; and British Columbia Research Centre for Children and Women's Health, Vancouver, British Columbia, Canada V5Z 4H4[3]

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A homologue of the *algC* gene, responsible for the production of a phosphoglucomutase (PGM) associated with LPS and alginate biosynthesis in *Pseudomonas aeruginosa*, *spgM*, was cloned from *Stenotrophomonas maltophilia*. The *spgM* gene was shown to encode a bifunctional enzyme with both PGM and phosphomannomutase activities. Mutants lacking *spgM* produced less LPS than the SpgM$^+$ parent strain and had a tendency for shorter O polysaccharide chains. No changes in LPS chemistry were obvious as a result of the loss of *spgM*. Significantly, however, *spgM* mutants displayed a modest increase in susceptibility to several antimicrobial agents and were completely avirulent in an animal model of infection. The latter finding may relate to the resultant serum sensitivity of *spgM* mutants which, unlike the wild-type parent strain, were rapidly killed by human serum. These data highlight the contribution made by LPS to the antimicrobial resistance and virulence of *S. maltophilia*.

Stenotrophomonas maltophilia is a gram-negative bacterium that is an important cause of nosocomial infections (17, 34). It has emerged as an important opportunistic pathogen in immunodeficient patients, including transplant recipients (38), cancer patients (33), and AIDS sufferers (19). However, the most frequent site of infection remains the lungs (4, 9, 20). In many cases, treatment of *S. maltophilia* is problematic owing to its high level of intrinsic resistance to multiple classes of antibiotics (56). A number of factors, including multidrug efflux pumps (1, 3, 65) and outer membrane impermeability (13, 37), likely contribute to the intrinsic antibiotic resistance of *S. maltophilia*. A key component of the outer membrane is lipopolysaccharide (LPS), and changes in LPS structure have been correlated with changes in resistance to a variety of antimicrobial agents (reviewed in reference 41).

Several groups have investigated LPS in *S. maltophilia* in an effort to assess its contribution to antimicrobial resistance in this organism (43–45, 60). *S. maltophilia* exhibits a temperature-dependent variation in susceptibility to several antibiotics, including aminoglycosides and polymyxin B (60), whose activities are known to be affected by LPS (44), but not quinolones, β-lactams, and chloramphenicol (44, 60). Temperature-dependent changes in outer membrane fluidity (44), LPS side-chain length (45) and, possibly, core phosphate content (43) appear to explain the temperature-dependent variation in aminoglycoside susceptibility, implicating LPS as a determinant of aminoglycoside resistance in this organism. Temperature-dependent changes in lipid A (44), outer membrane proteins (45), or 3-deoxy-D-manno-octulosonic acid (43) have not been observed.

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Queen's University, Kingston, Ontario, Canada K7L 3N6. Phone: (613) 533-6677. Fax: (613) 533-6796. E-mail: poolek@post.queensu.ca.

Besides the focus on its contribution to antimicrobial resistance, LPS has received a great deal of attention with regard to its role in the development and maintenance of a productive infection. A loss of O polysaccharide (O-PS) in *Burkholderia pseudomallei*, for example, by mutation of the rhamnose biosynthesis pathway, reduces the virulence of this organism in an animal model of infection (15). Similarly, defects in O-PS biosynthesis in *Brucella abortus* (55) and *Pseudomonas aeruginosa* (23) result in avirulent isolates of these organisms. Even more subtle changes in O-PS structure can have a dramatic effect on virulence. For example, clinical isolates of *Salmonella enterica* serovar Enteritidis have a much higher level of glucosylation branching of their O-PS than do avirulent isolates (42). The core polysaccharide also plays an important role in LPS structure and, ultimately, virulence (23, 58, 63). Truncation of LPS in the core of both *P. aeruginosa* (23) and *Bordetella bronchiseptica* (59) results in avirulent isolates, while decreases in core phosphorylation lead to mutants of *S. enterica* (63) and *P. aeruginosa* (58) that are less virulent in animal models.

With much of the focus to date on *S. maltophilia* LPS structure (39, 44, 45, 61), virtually nothing is known about the enzymes involved in the biosynthesis of LPS for this organism. Similarly, although several studies have addressed mechanisms of antibiotic resistance in this organism (2, 3, 36, 65), the contributions that LPS makes to antibiotic resistance and to pathogenesis remain largely unexplored. To gain some insight into the roles of LPS in antibiotic resistance and virulence, we sought to identify genes involved in LPS biosynthesis in *S. maltophilia*. Given the central role of *P. aeruginosa* AlgC, a phosphoglucomutase (PGM), in the production of alginate (66), LPS (12, 66), and rhamnolipids (40) as well as its impact on antibiotic susceptibility (31) and virulence (23), we focused our efforts on the recovery of an AlgC homologue in *S. maltophilia*. We report here the cloning and characterization of

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)[a]	Reference or source
Strains		
P. aeruginosa		
PAO1	*P. aeruginosa* prototroph	24
AK1012	PAO1 *algC*; A^- B^{-b}	29 12
S. maltophilia		
K1014	Clinical isolate; Kingston General Hospital	This study
K2049	K1014 *spgM*::*kan*[c]	This study
K1199	Reference strain ULA-511	18
K2048	K1199 *spgM*::*kan*[c]	This study
Plasmids		
pEX18Tc	Gene replacement vector; Mob^+ *sacB* Tc^r	27
pRK415	Low-copy-number, broad-host-range cloning vector carrying MCS downstream of P*lac*; Tc^r	32
pUC4K	Source of kanamycin resistance cassette (*kan*); Ap^r	Pharmacia
pNZ49	pMMB66EH derivative carrying the *P. aeruginosa algC* gene; Ap^r	66
pUC18	High-copy-number cloning vector; P*lac* Ap^r	62
pGAM01	pEX18Tc containing an 800-bp fragment of *spgM* disrupted by a *kan* cassette at a unique *Sal*I site	This study
pGAM02	pUC18 derivative carrying a ca. 1.8-kb *spgM* gene	This study
pGAM03	pRK415 derivative carrying a 1,963-bp fragment containing the *spgM* gene	This study

[a] Ap^r, ampicillin resistance; Tc^r, tetracycline resistance; MCS, multiple cloning site; P*lac*, *lac* promoter.
[b] AK1012 produces neither A-band nor B-band polysaccharide.
[c] The K1199 *spgM* gene is interrupted by the *kan* cassette at a unique *Sal*I site.

spgM and the impact of its loss on LPS structure, drug susceptibility, and virulence in *S. maltophilia*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *S. maltophilia* strains were grown in Luria-Bertani (LB) broth or on LB agar (Luria broth base; Difco) supplemented with 2 g of NaCl/liter. Standard agar contained 1.5% (wt/vol) Bacto Agar (Difco), while top agar contained 0.75% (wt/vol) Bacto Agar. Bacteria were cultured at 37°C unless otherwise indicated. Plasmids pRK415, pEX18Tc, pUC18, pNZ49, and pUC4K (and their derivatives) were maintained in *E. coli* with appropriate antibiotic selection (pRK415 and pEX18Tc, 10 μg of tetracycline per ml; pNZ49 and pUC18, 100 μg of ampicillin per ml; and pUC4K, 40 μg of kanamycin per ml).

Molecular biology techniques. Restriction endonuclease digestions, ligations, transformations, and colony blot hybridizations were performed as described by Sambrook et al. (47). DNA fragments used in cloning were extracted from agarose gels with a Qiaex II kit (Qiagen, Mississaugua, Ontario, Canada) according to the manufacturer's instructions. DNA probes were labeled with digoxigenin-coupled dUTP by using a Dig High Prime II kit (Boehringer-Mannheim, Laval, Quebec, Canada), and Southern hybridization was performed as specified by the manufacturer under low-stringency conditions (68°C with 0.1% sodium dodecyl sulfate [SDS]– 0.5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] [pH 7.0] posthybridization washes). Sequencing of *spgM* was performed at Cortec DNA Service Laboratories Inc. (Kingston, Ontario, Canada).

Cloning of the *S. maltophilia spgM* gene. A series of genomic DNA digests of *S. maltophilia* strains K1014 and K1199 were screened with a *P. aeruginosa algC* probe at 65°C for 12 h under low-stringency conditions. The *algC* probe was generated by excising from plasmid pNZ49 an ~2-kb *Hin*dIII-*Sst*I fragment which was then used as a template for the Dig High Prime II kit. Strong positive signals were observed at ~2.5 kb for *Ava*I digests and at ~1.8 and ~3.3 kb for *Msc*I digests. Corresponding regions of appropriately digested chromosomal DNAs were subsequently excised from agarose gels and purified by using the Qiaex II kit. The purified DNAs were subsequently cloned into *Ava*I- or *Hin*cII-digested pUC18 as appropriate and transformed into calcium-competent *E. coli* DH5α. Ampicillin-resistant colonies were screened with the *algC* probe by colony blot hybridization as described above. One positive clone, designated pGAM02, carried an *Msc*I insert of ~1.8 kb; upon sequencing, this insert was found to contain an open reading frame, dubbed *spgM*, of 1,344 bp. This size was consistent with the sizes of previously reported PGM genes (35, 66). A 1,963-bp fragment was excised from pGAM02 by *Hin*dIII-*Bam*HI digestion and then cloned into *Hin*dIII-*Bam*HI-restricted pRK415 to produce pGAM03 for use in *spgM* complementation studies with *S. maltophilia*.

Insertional inactivation of the *spgM* gene. An *S. maltophilia spgM* chromosomal knockout was constructed by using gene replacement vector pEX18Tc. First, a 757-bp internal fragment was excised from *spgM* by using *Eco*RV and *Msc*I and then cloned into *Hin*cII-digested pEX18Tc. Next, a 1,252-bp kanamycin resistance cassette was excised from plasmid pUC4K by *Sal*I digestion and then inserted into a unique *Sal*I (position 517) site within the pEX18Tc-borne *spgM* gene to form plasmid pGAM01. This vector was introduced into *E. coli* S17-1 and mobilized into *S. maltophilia* strains K1199 and K1014 via conjugation as described previously (64). Transconjugants were selected on LB agar containing 40 μg of tetracycline per ml and 10 μg of norfloxacin per ml (for counterselection). Multiple individual colonies were streaked on LB agar containing 10% (wt/vol) sucrose and then incubated at 37°C overnight. On the following day, sucrose-resistant colonies were selected and screened for kanamycin resistance cassette inactivation of the chromosomal *spgM* gene in kanamycin nutrient broth. Southern hybridization ultimately confirmed the disruption of the *spgM* gene.

LPS PAGE analysis. Small-scale LPS preparations were obtained by small-scale phenol extractions of LB medium cultures of *S. maltophilia* strains K1014 and K2049. Briefly, 1 ml of mid-log-phase culture (2.5×10^7 cells) grown in LB broth at 37°C was harvested by centrifugation at 10,000 × *g* for 2 min at room temperature. The cell pellet was resuspended in 200 μl of water, and an equal volume of 90% (vol/vol) Tris-HCl-buffered phenol (100 mM Tris-HCl [pH 8.0]) was added. The samples were heated for 10 min at 70°C and then cooled for 10 min on ice. Following centrifugation at 6,500 × *g* for 2 min at room temperature, the upper aqueous layer was removed and reextracted with an equivalent volume of chloroform. The aqueous phase was recovered and frozen (−20°C) until needed. A modified polyacrylamide gel electrophoresis (PAGE) protocol, in which SDS was omitted from both stacking and resolving gels, was used to visualize LPS (14). To resolve core polysaccharide regions of LPS, a Tricine-PAGE protocol was used as described elsewhere (50). Silver staining of LPS gels was done by the method of Tsai and Frasch (54).

Enzyme assays. PGM activity was assessed by using whole-cell lysates according to a previously published protocol (57). Briefly, 7.5 ml of an overnight culture grown in LB broth (with appropriate antibiotic selection when necessary) was inoculated into 750 ml of LB broth, and the cells were grown for 6 h at 37°C with shaking. Cells were harvested by centrifugation at 6,000 × *g* and cooled to 4°C. The cell pellet was washed with 50 ml of precooled 0.85% (wt/vol) NaCl and resuspended in 5 ml of precooled sonication buffer (50 mM morpholinepropanesulfonic acid [MOPS], 1 mM dithiothreitol [DTT], 3 mM EDTA, 1% [vol/vol] Trition X-100 [pH 7.0]). Cells were disrupted by sonication (three 45-s pulses) with a VibraCell set at 40 U (Sonics and Materials, Danbury, Conn.), and a crude extract (supernatant) was obtained following centrifugation at 150,000 × *g* for 1 h. Samples were prepared fresh for all assays and were stored on ice until needed. A 1-ml reaction mixture consisted of 15 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM glucose-1,6-bisphosphate, 1 mM glucose-1-phosphate, 1 U of glucose-6-phosphate dehydrogenase, and 1 mM β-$NADP^+$. The reaction mixture was allowed to equilibrate at room temperature for 5 min, and then the reaction was initiated by the addition of 10 μl of whole-cell lysate. The progress of the reaction was monitored at 340 nm. Phosphomannomutase (PMM) activity was assayed in a reaction similar to that described for the PGM activity assay, except that the above reaction mixture was supplemented with 1 U each of phosphoglucose isomerase and phosphomannose isomerase, while the substrate glucose-1-phosphate was replaced with mannose-1-phosphate (1 mM). Initial rates were measured from progress curves for reactions with and without the substrates glucose-1-phosphate (for PGM assays) and mannose-1-phosphate (for PMM assays). Slopes were converted to rates by using the molar extinction coefficient for β-$NADP^+$ (6,300 liters/mmol).

Large-scale preparations of LPS and O-PS. *S. maltophilia* strains K1014 and K2049 were grown for 12 h at 37°C in 50-liter cultures of LB broth. The wet cell pellets (51 and 35 g, respectively) were resuspended in 500 ml of tap water and extracted with 50% (vol/vol) aqueous phenol for 15 min at 70°C. Following the

addition of 2 volumes of water, the mixture was subjected to low-speed centrifugation (5,000 × *g*) for 30 min at 4°C to remove particulate matter. Both the aqueous and the phenol layers were removed and dialyzed separately against running tap water for 2 days until all traces of phenol were removed. The samples (aqueous and phenol) were pooled and lyophilized. The lyophilized dialysate was dissolved in 20 ml of deionized distilled water and treated sequentially with 200 μg of DNase and 50 μg of RNase for 1 h at 37°C, after which 200 μg of proteinase K was added and incubation was continued for a further 3 h at 37°C. The enzyme-treated sample was subjected to ultracentrifugation at 100,000 × *g* for 16 h at 4°C to yield LPS as a viscous gel pellet. This pellet was subsequently resuspended in 50 ml of deionized distilled water and lyophilized (yields, 685 mg for strain K1014 and 167 mg for strain K2049). To separate O-PS from lipid A, samples of K1014 LPS and K2049 LPS (295 and 69 mg, respectively) were dissolved in 50 ml of 2% (vol/vol) acetic acid and hydrolyzed at 100°C for 2 h. The hydrolysate was subjected to low-speed centrifugation (6,000 × *g* for 30 min) to remove insoluble lipid A material. The supernatant was lyophilized to dryness, and the material was dissolved in 5 ml of 0.05 M pyridinium acetate (pH 4.6). This mixture then was applied to a Sephadex G-50 (1 m by 2.5 cm) column preequilibrated with the same buffer. The eluate was monitored by using the refractive index, and fractions (10 ml) were analyzed colorimetrically for neutral glycose by the phenol-sulfuric acid method (16) and for amino glycoses (22).

Sugar composition and linkage analyses. To elucidate the compositions and linkages of sugars in the O-PS of *S. maltophilia*, strains K1014 and K2049 were analyzed by standard alditol acetate and permethylated alditol acetate protocols (11). Samples of K1014 or K2049 O-PS were dissolved in 1 ml of 4 M trifluoroacetic acid and hydrolyzed by heating at 100°C for 4 h. The hydrolysate was reduced with a molar excess of sodium borodeuteride overnight in the dark. Acetylation was achieved with 2 ml of acetic anhydride at 100°C for 2 h. The alditol acetate derivatives were analyzed by gas-liquid chromatography (GLC) with a Hewlett-Packard 5890A chromatograph equipped with a 30-m DB-17 silica capillary column (180 to 240°C at 2°C/min). Linkage analysis was performed by using the NaOH-dimethyl sulfoxide (DMSO)-CH_3I procedure (11). Briefly, 20-mg samples were dissolved in 5 ml of anhydrous DMSO, and powdered NaOH-methyl iodide (5 ml) was added. Following vigorous stirring for 2 h at room temperature, the resultant permethylated alditol acetate derivatives were analyzed by GLC-mass spectroscopy with a Hewlett-Packard 5985B chromatograph equipped with a DB-17 column (isothermally 190°C) in the electron impact mode.

One-dimensional ^1H nuclear magnetic resonance (NMR) experiments were recorded on a Bruker AMX 500 spectrometer at 298 K by using Varian software. Samples were lyophilized three times with D_2O (99.9%) prior to performance of the NMR experiments. The partially deuterated water peak was used as the reference at 4.786 ppm.

Antimicrobial susceptibility testing. The susceptibilities of *S. maltophilia* strains to a number of antimicrobials agents were assessed with serial twofold dilutions of each antimicrobial agent in LB broth and with an inoculum of 10^5 organisms as described previously (64). Bacterial inocula were prepared by dilution of overnight cultures grown in LB broth at 37°C.

Rat lung infection model. The virulence of *S. maltophilia* strains K1014 and K2049 and their *spgM* derivatives was assessed by using a rat model of chronic respiratory infection with quantitative pathologic and bacteriologic results as measures of the productivity of infection (51).

Nonopsonic phagocytosis, phagocytic killing, and serum sensitivity assays. Human neutrophils were isolated as described previously (25) and used in nonopsonic phagocytosis and phagocytic killing assays by a previously described protocol (52), with some modifications. Experiments were performed at 37°C. For the phagocytic assay, 1 ml of ice-cold phosphate-buffered saline was added to each tube following 1 h of incubation. Cells were fixed with 2% (vol/vol) formaldehyde in phosphate-buffered saline, cytospun onto glass slides, fixed with 100% (vol/vol) methanol, and stained with an excess of 5% (wt/vol) Giemsa stain. Serum sensitivity assays were completed as detailed below. LB broth-grown overnight cultures of *S. maltophilia* strains K1014, K2049, and K2049 harboring pGAM03 were diluted 1:100 in sterile 0.85% (wt/vol) NaCl. A 100-μl sample of each diluted culture was added to a prechilled sterile glass culture tube. Following the addition of an equal volume of freshly drawn human serum, the bacteria were incubated at 37°C. Samples of 5 μl were removed at 0, 5, 10, 20, and 40 min, and appropriate dilutions were plated on LB agar plates (to determine viable cell counts).

Chemicals. Norfloxacin, kanamycin, tetracycline, vancomycin, ampicillin, nalidixic acid, acetic anhydride, trifluoroacetic acid, sodium borodeuteride, NaOH, methyl iodide (CH_3I), MOPS, DTT, EDTA, and DMSO were obtained from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada). Polymyxin B was obtained from ICN Biomedicals Inc. (Aurora, Ohio). α-D-Glucose-1,6-diphosphate, α-D-mannose-1-phosphate, α-D-glucose-1-phosphate, β-NADP, glucose-6-phosphate dehydrogenase, phosphomannose isomerase, and phosphoglucose isomerase were obtained from Sigma (St. Louis, Mo.).

Nucleotide sequence accession number. The *spgM* sequence has been deposited with GenBank under accession number AY179964.

TABLE 2. PGM and PMM activities of *S. maltophilia* and *P. aeruginosa*

Strain	Activity (pmol/min/μg)[a] of:	
	PGM	PMM
S. maltophilia		
K1199	206	58
K2048	5	5
K2048(pGAM03)[b]	705	105
K1014	261	—
K2049	0.7	—
K2049(pGAM03)	170	—
P. aeruginosa		
PAO1	144	—
AK1012[c]	6.5	—
AK1012(pGAM03)	745	—

[a] Rate of reaction calculated in duplicate by coupling assays as described in the text. Data are the means of three separate determinations. —, values were not determined.
[b] pGAM03 is a pRK415 derivative carrying *spgM*.
[c] AK1012 is an *algC* rough mutant of PAO1.

RESULTS

Cloning of the *S. maltophilia spgM* gene. In a search for key genes involved in LPS biosynthesis and, thus, likely to contribute to LPS-associated antimicrobial resistance and virulence, restriction endonuclease digests of *S. maltophilia* strain K1199 chromosomal DNA were screened by Southern hybridization with the *P. aeruginosa algC* gene as a probe. Sequencing of the cloned DNA revealed a complete open reading frame of 1,344 bp. The putative translation product from the open reading frame is a protein of 448 amino acids with a predicted molecular mass of 48,707 Da and a pI of 5.69. The deduced product showed homology to the XanA PGM of *Xanthomonas campestris* (79.9% identity) (35), the PGM of *Neisseria gonorrhoeae* (30.7% identity) (48), and the AlgC PGM of *P. aeruginosa* (29.9% identity) (66). The gene was thus dubbed *spgM* (*Stenotrophomonas* PGM).

SpgM displays PGM and PMM activities. Homology studies suggested that SpgM was an enzyme with PGM activity. To assess this idea, PGM assays were carried out with whole-cell lysates of wild-type *S. maltophilia* strains K1199 and K1014 as well as their *spgM* counterparts, K2048 and K2049, respectively. The two wild-type strains were found to have approximately equivalent levels of PGM activity, while their *spgM* derivatives had 40-fold (K2048) and 370-fold (K2049) less PGM activity (Table 2). As expected, the cloned *spgM* gene (on plasmid pGAM03) restored the PGM activity in the *spgM* mutants (Table 2). Similar studies demonstrated that pGAM03 was also capable of complementing *P. aeruginosa algC* mutant strain AK1012, restoring PGM activity in this strain as well (Table 2). These data indicate that *spgM* encodes an enzyme with PGM activity.

Given the high overall levels of identity to XanA, NpgM, and AlgC, a subclass of bifunctional enzymes that exhibit PMM

activity in addition to PGM activity, the PMM activity of SpgM was also investigated. Cell lysates prepared from wild-type strain K1199 indeed displayed PMM activity that was, however, fourfold lower than the PGM activity of this strain (Table 2). Lysates prepared from *spgM* mutant K2048 displayed markedly reduced PMM activity that was <10% the parental level (Table 2). Again, introduction of the cloned *spgM* gene on plasmid pGAM03 restored PMM activity (Table 2). These data suggest that SpgM is a functional hexose phosphate mutase. At saturating substrate concentrations, SpgM demonstrates a fourfold-higher rate of reaction for glucose than for mannose, although no statement as to substrate specificity can be made at this time.

Involvement of SpgM in LPS biosynthesis. To assess the impact of *spgM* loss in strains K2048 and K2049 on LPS synthesis, small-scale preparations of LPS from wild-type and *spgM* strains were resolved on silver-stained polyacrylamide gels. While wild-type *S. maltophilia* K1014 LPS exhibited a broad range of sizes, including many higher-molecular-weight species (Fig. 1A, lane 1), its *spgM* mutant strain, K2049, demonstrated a smaller range of LPS sizes and substantially lower molecular weights (Fig. 1A, lane 2). Although *S. maltophilia* strain K1199 produced LPS with a much more limited range of sizes and with markedly lower molecular weights than did K1014, its *spgM* derivative, K2048, also showed a trend toward lower-molecular-weight species (data not shown). The cloned *spgM* gene on pGAM03 restored the LPS banding pattern of K2049 to that of the wild type (Fig. 1A, lane 3). Resolution of the core region of LPS with Tricine gels failed to reveal any differences between the $SpgM^+$ (Fig. 2B, lanes 1 and 3) and $SpgM^-$ (Fig. 2B, lane 2) strains. In contrast, *P. aeruginosa algC* mutant strain AK1012 synthesized only a partial core (Fig. 2B, lane 5) compared to its wild-type counterpart (Fig. 1B, lane 4). Interestingly, introduction of the *S. maltophilia spgM* gene on pGAM03 into AK1012 restored the complete core structure (Fig. 1B, lane 6). These data demonstrate that SpgM is readily able to replace AlgC with respect to LPS biosynthesis in *P. aeruginosa*.

To assess whether there were chemical changes in the LPS of an *spgM* mutant, large-scale preparations of LPS samples from wild-type *S. maltophilia* strain K1014 and its *spgM* counterpart, K2049, were purified and analyzed. Strain K1014 was obtained as a clinical isolate with an unknown serotype; thus, its LPS chemical structure was undefined. The final yields of Sephadex G-50-purified O-PS for strains K1014 and K2049 were 87 mg (1.3%) and 17 mg (0.5%), respectively. It is clear that the yield of O-PS from *S. maltophilia* strain K2049 was substantially smaller than that from its parent. Initial analysis of the O-PS from K1014 revealed a hexose content of 2.14 rhamnose, 1.01 fucose, and 1 glucose molecules (Fig. 2A). A similar rhamnose/fucose/glucose ratio of 2.34:1:1.22 was also observed for the *spgM* knockout strain (Fig. 2B), indicating that the mutant was able to produce a O-PS with the same chemical structure as that of the wild type. Linkage analysis of both K1014 O-PS and K2049 O-PS indicated the presence of →3)-Rha*p*-(1→, →4)-Rha*p*-(1→, →3)-Fuc*p*-(1→ and Glc*p*-(1→. This structure was found to correspond to the O-PS of *S. maltophilia* serotype 19. Other constituents detected in smaller amounts included mannose, galactose, and *N*-acetylgalactosamine, which had all been previously identified as components of the *S. maltophilia* core oligosaccharide (39). Also detected in very small quantities was L-glycero-α-D-mannoheptose, which was previously reported to be absent from the core of *S. maltophilia* (39). To confirm the chemical analysis of K1014 and K2049, purified O-PSs from both strains were analyzed by ^1H NMR spectroscopy and again shown to be identical (data not shown).

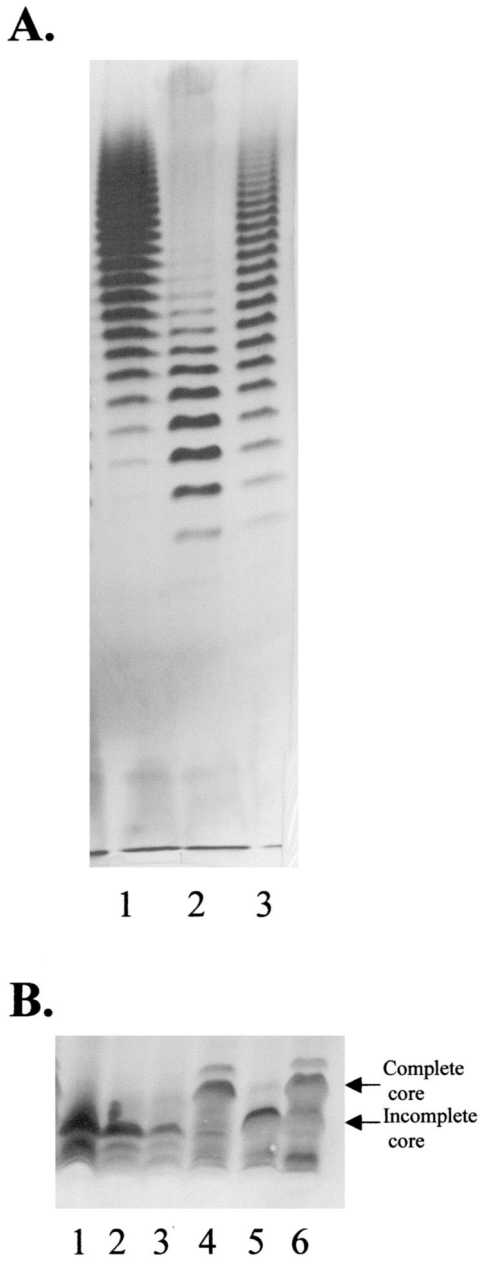

FIG. 1. SDS-PAGE analysis of O-PS (A) and core LPS (B) from proteinase K-digested whole-cell lysates of *S. maltophilia*. (A) Lane 1, wild-type *S. maltophilia* strain K1014; lane 2, *S. maltophilia spgM* mutant K2049; lane 3, *S. maltophilia* K2049 harboring plasmid pGAM03. (B) Lane 1, wild-type *S. maltophilia* K1014; lane 2, *S. maltophilia spgM* mutant K2049; lane 3, *S. maltophilia* K2049 harboring plasmid pGAM03; lane 4, *P. aeruginosa* PAO1; lane 5, *P. aeruginosa* AK1012 (PAO1 *algC*); lane 6, *P. aeruginosa* AK1012 harboring pGAM03.

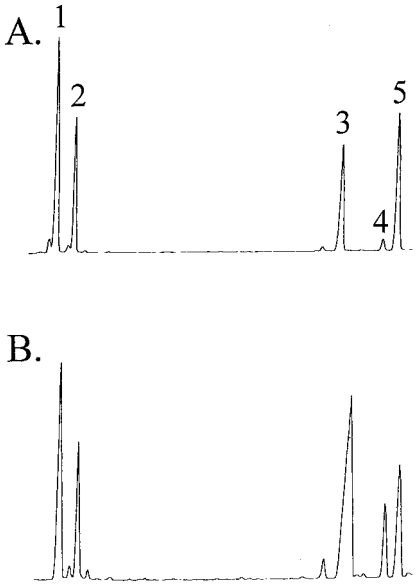

FIG. 2. Separation of monosaccharides obtained by hydrolysis of *S. maltophilia* O-PS. The identities of the peaks were obtained by analysis of carbohydrate standards. The peaks are as follows: peak 1, rhamnose; peak 2, fucose; peak 3, inositol (internal standard); peak 4, mannose; peak 5, glucose. (A) GLC alditol acetate trace of wild-type *S. maltophilia* strain K1014. The rhamnose/fucose/glucose ratio for K1014 was determined to be 2.14:1.01:1. (B) GLC alditol acetate trace of *S. maltophilia spgM* mutant K2049. The rhamnose/fucose/glucose ratio for K1014 was determined to be 2.34:1:1.22. Ratios were determined by integration of the area under the peaks for all carbohydrates detected in GLC alditol acetate traces.

Influence of the *spgM* mutation on susceptibility to antimicrobial compounds. LPS is an important determinant of antimicrobial resistance in a number of gram-negative organisms, including *S. maltophilia* (8, 31, 43–45, 55, 58, 59, 63). To assess the impact, then, of the *spgM* mutation and the attendant changes in LPS on antimicrobial susceptibility in *S. maltophilia*, the *spgM* mutant strains K2048 and K2049 and their parent strains were tested for susceptibility to antimicrobial compounds known to interact with LPS, including aminoglycosides, vancomycin, quinolones, and the cationic peptides polymyxin B and polymyxin E. As shown in Table 3, the *spgM* mutant strains exhibited a two-to fourfold increase in susceptibility to polymyxin B, polymyxin E, nalidixic acid, and gentamicin relative to their parent strains. Interestingly, however, the *spgM* mutant strains also exhibited a two- to fourfold increase in resistance to vancomycin relative to their parent strains (Table 3).

TABLE 3. Influence of *spgM* on the antimicrobial susceptibility of *S. maltophilia*

S. maltophilia strain	MIC (μg/ml)[a]				
	Nalidixic acid	Gentamicin	Vancomycin	Polymyxin B	Polymyxin E
K1014	37.5	128	175	20	37.5
K2049	18.5	64	750	10	18.5
K2049(pGAM03)	37.5	128	350	20	37.5
K1199	37.5	64	175	20	75
K2048	18.5	32	375	10	18.5
K2048(pGAM03)	37.5	64	175	20	37.5

[a] With the exception of gentamicin MICs, which were determined in nutrient broth, all MICs were determined in LB broth.

TABLE 4. Influence of *spgM* on the quantitative bacteriologic and pathologic results for *S. maltophilia* in rat lungs

Strain	Mean ± SD result of the following analysis[a]	
	Bacteriologic	Pathologic
K1014	1,400 ± 600	32.1 ± 12.5
K2049	ND[b]	3.3 ± 0.9[b]
K2049(pGAM03)	1,350 ± 663	30.7 ± 11.6

[a] Lungs were excised 7 days postinoculation and analyzed for either viable cell counts (quantitative bacteriologic analysis) or histopathological changes (quantitative pathologic analysis). ND, none detected.

[b] Significantly different from values for K1014 and K2049 (P value, <0.01, as determined by a paired t test with the Bonferroni correction for multiple comparisons).

Influence of the *spgM* mutation on virulence. Given the previously demonstrated importance of LPS structure to virulence in a number of gram-negative pathogens (23, 55, 59), it was of interest to determine whether *spgM* mutants were at all compromised with regard to *S. maltophilia* virulence. Using a rat lung model of infection, it was shown that *spgM* mutant strain K2049 was completely unable to colonize rat lungs, while substantial numbers of its wild-type parent strain, K1014, were recovered from rat lungs 7 days postinfection (Table 4). The introduction of the *spgM* gene on plasmid pGAM03 into K2049 did, however, restore its ability to colonize rat lungs (Table 4), clearly demonstrating the importance of *spgM* and, thus, wild-type LPS in colonization. Examination 7 days postinoculation of lung tissues from rats infected with K1014 or K2049 harboring pGAM03 showed evidence of histopathological changes (Table 4), including brochiectasis of airway epithelium and inflammatory infiltrates consisting of proteinaceous exudates and polymorphonuclear leukocytes (data not shown). No histopathological changes were observed for *spgM* mutant K2049 (Table 4) ($P < 0.01$). Similar results were obtained for *S. maltophilia* strain K1199 and its *spgM* knockout strain, K2048 (data not shown).

To better understand the reasons for the compromised virulence of the *spgM* mutant, the susceptibility of wild-type strain K1014 and mutant strain K2049 to host defenses, including phagocytosis and complement-mediated lysis, was assessed. While the parent and the mutant were both efficiently phagocytosed by neutrophils, they were also both resistant to killing (data not shown). Unlike parent strain K1014, however, which was resistant to complement-mediated cell killing (100% survival after 40 min of exposure to human serum) (Fig. 3), *spgM* deletion strain K2049 was susceptible to complement, exhibiting a viable cell count of 0 after 10 min of exposure to human serum (Fig. 3). Introduction of the cloned *spgM* gene on plasmid pGAM03 substantially although only partially restored the resistance to complement-mediated killing of K2049, with viable cell counts of 65% that of the wild type at 10 min and 37% that of the wild type at 40 min (Fig. 3). While the restoration of serum resistance by the plasmid-borne *spgM* gene was only partial, these results reflected the only partial restoration of PGM activity provided by pGAM03-encoded SpgM in strain

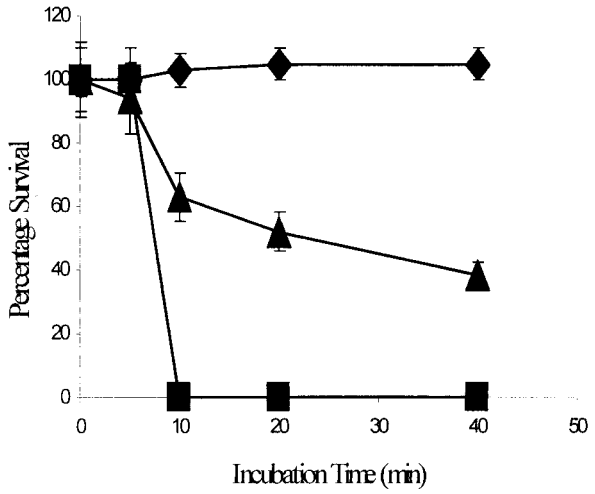

FIG. 3. Serum sensitivities of *S. maltophilia* strains K1014 (◆), K2049 (■), and K2049 carrying SpgM-encoding plasmid pGAM03 (▲). Bacterial cultures were mixed with an equal volume of human serum, and viable cell counts were determined as a function of time of incubation at 37°C. Data are reported as a percentage of the original cell count at 0 min. The data points are the means and standard deviations for three plates and are from one of three independent experiments with similar results.

K2049 (65% the activity of SpgM$^+$ wild-type strain K1014) (Table 2).

DISCUSSION

The studies reported here describe the cloning and characterization of a PGM gene, *spgM*, from the opportunistic human pathogen *S. maltophilia*. Enzyme assays confirmed that SpgM possesses both PGM and PMM activities, reminiscent of the PGMs of *P. aeruginosa* (AlgC) (66), *N. gonorrhoeae* (48), *X. campestris* (XanA) (35), and *B. bronchiseptica* (59). These enzymes form a class of PGM and PMM enzymes within the larger superfamily of hexose phosphate mutases, which include PGMs, PMMs, and phosphoglucosamine mutases.

Chemical analysis and ^{1}H NMR of the O-PS of wild-type strain K1014 and the O-PS of its *spgM* mutant strain, K2049, identified as major components L-rhamnose, D-glucose, and D-fucose at a ratio of approximately 2:1:1. This chemical composition is consistent with a previously reported structure for *S. maltophilia* serotype 19 (61). This is the third most prevalent serotype, accounting for 13.8% of all identifiable isolates (49), and is therefore of clinical importance. Other minor constituents were identified, including mannose, galactose, *N*-acetylgalactosamine, and L-glycero-α-D-mannoheptose, which are believed to be components of the core polysaccharide. The latter species was previously reported to be absent from the *S. maltophilia* core (39). Nonetheless, core polysaccharide appeared unaffected in *spgM* mutant K2049, indicating that the elimination of *spgM* did not adversely affect the core structure in *S. maltophilia*.

LPS structure has been implicated as an important virulence factor in numerous gram-negative organisms (23, 55, 59), although its contribution to virulence in *S. maltophilia* remains unresolved. It is clear that the studies presented here indicate that LPS is an important determinant of virulence in this organism and, moreover, that SpgM plays an important role in maintaining the virulence-dependent LPS structure. The reduced virulence of *S. maltophilia spgM* mutants may be related, at least in part, to an increased sensitivity to complement-mediated lysis although clearly not to phagocytic killing. Studies of other gram-negative organisms, including *Bordetella* (59), *Pseudomonas* (23), and *Brucella* (55) species, have also confirmed the importance of a PGM with regard to LPS structure and virulence.

To assess the contribution of *spgM* to membrane structure, the outer membrane LPS barrier of *S. maltophilia* was probed with antimicrobial compounds nalidixic acid, vancomycin, polymyxin, and gentamicin. The outer membrane of *S. maltophilia* serotype 19 has been determined to be extremely hydrophilic (30). Elimination of *spgM* in a seroptype 19 strain of *S. maltophilia* renders the organism approximately twofold more susceptible to nalidixic acid, with no change in susceptibility to the hydrophilic fluoroquinolones ciprofloxacin and norfloxacin (data not shown). Apparently, this hydrophobic quinolone has a moderately increased ability to permeate the outer membrane of *S. maltophilia* in the LPS mutant. Given the expected route of entry for nalidixic acid (the lipid bilayer) (6, 26), it appears that the surface hydrophobicity of the *spgM* mutant is higher than that of the wild type. O-PS mutants of *Serratia marcescens* also exhibit a twofold increase in susceptibility to nalidixic acid with no change in susceptibility to hydrophilic fluoroquinolones (6). Conversion of long-chain O-PS in wild-type LPS of *S. maltophilia* to a shorter O-PS likely permits more efficient partitioning of nalidixic acid into the outer membrane, leading to the observed increase in susceptibility to this compound.

Surprisingly, the change in O-PS structure seen in the *spgM* mutant renders the bacterium noticeably more resistant to the glycopeptide vancomycin. While a possible increase in surface hydrophobicity in the mutant might explain the reduced uptake of this hydrophilic agent (vancomycin is thought to cross the outer membrane by transit through the lipid bilayer), it is also conceivable that the change in the O-PS structure eliminated or altered the cell surface binding of vancomycin. The observation that the loss of *spgM* produces an increase in susceptibility to cationic antibiotics is reminiscent of earlier studies with *S. maltophilia*, where resistance to aminoglycosides was also correlated with O-PS levels (45). Similarly, *pgm* mutants of *B. bronchiseptica* (59) and *B. abortus* (55) display enhanced susceptibility to cationic antimicrobial agents.

The elimination of the *spgM* gene appeared to alter the modality of the O-PS side chains in *S. maltophilia*, reminiscent of changes observed for *rol* mutants in *Salmonella* (5) and *P. aeruginosa* (10), yet the amplitude of the banding appeared unaffected. While this finding is somewhat surprising, similar observations have been noted elsewhere. Loss of the dTDP-L-rhamnose synthase in *Azorhizobium caulinodans* leads to a virtually identical alteration in the modality of the O-PS side chains, and the authors suggested the existence of an alternative rhamnose synthase with sufficient activity to account for the O-PS synthesized in a dTDP-L-rhamnose synthase mutant (21). It is clear that SpgM does not play a direct role in regulating O-PS chain length, which is typically attributed to products of the *wzy* (O-antigen polymerase) and *wzz* (O-anti-

gen chain-length regulator) genes (46). More likely, the loss of SpgM activity and the attendant decline in glucose-1-phosphate levels compromise the production of the activated rhamnose and glucose sugars that are incorporated into *S. maltophilia* serotype 19 LPS (glucose-1-phosphate is required for the synthesis of these activated sugars). With a limited pool of these sugars available for incorporation into O-PS, it is perhaps not unexpected that full-length O-PS is not synthesized in *spgM* mutants. In any case, residual PGM activity must be present in *spgM* mutants to provide for the observed O-PS, perhaps due to the presence of an additional hexose phosphate mutase in this organism. Interestingly, the phosphoglucosamine mutase recently identified in *P. aeruginosa* shows substantial active-site homology with SpgM and also has been demonstrated to have modest PGM activity (53). The yeast *Saccharomyces cerevisiae* has two PGM genes, *pgm1* and *pgm2*, that when disrupted impair the ability of the organism to grow on galactose as the sole carbon source. Complementation studies demonstrated that a cloned PMM (7) or *N*-acetylglucosamine phosphate mutase (28) could complement *pgm1*/*pgm2* knockout strains. In all likelihood, *S. maltophilia* expresses a similar hexose phosphate mutase, which might partially compensate for lost PGM activity. While this alternate PGM activity must exist, likely it is not sufficient to accommodate the high metabolic load required to synthesize glucose- and rhamnose-rich O-PS (one glucose and two rhamnose molecules per repeating unit, all derived from glucose-1-phosphate), as is the case in *S. maltophilia* K1014.

The observation that the elimination of *spgM* leads to an alteration in the modality of O-PS side chains, from a distribution of high-molecular-weight fragments to relatively small O-PS units, is, to our knowledge, unique within the gram-negative family of PGMs. Elimination of the PGMs from other gram-negative organisms produced either a total loss of O-PS, as in *P. aeruginosa algC* (12), *B. bronchiseptica pgm* (59), and *B. abortus pgm* (55) mutants, or had no effect whatsoever on LPS synthesis (e.g., *xanA* mutants of *X. campestris*) (35). Whereas an *spgM* mutation has only a modest effect on *S. maltophilia* LPS, the alteration in this macromolecule is sufficient to significantly impact virulence. Indeed, despite differences in the impact of *pgm* mutations on LPS structure in a number of gram-negative pathogens, PGM^- mutants appear to be universally less virulent than their wild-type counterparts. Thus, PGMs may represent an attractive novel drug target for the development of antimicrobial agents specific for gram-negative organisms.

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