The Role of Galacturonic Acid in Outer Membrane Stability in
Klebsiella pneumoniae*

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In most members of the Enterobacteriaceae, including Escherichia coli and Salmonella, the lipopolysaccharide core oligosaccharide backbone is modified by phosphoryl groups. The negative charges provided by these residues are important in maintaining the barrier function of the outer membrane. Mutants lacking the core heptose region and the phosphate residues display pleiotropic defects collectively known as the deep-rough phenotype, characterized by changes in outer membrane structure and function. Klebsiella pneumoniae lacks phosphoryl residues in its core, but instead contains galacturonic acid. The goal of this study was to determine the contribution of galacturonic acid as a critical source of negative charge. A mutant was created lacking all galacturonic acid by targeting UDP-galacturonic acid precursor synthesis through a mutation in glaKp. GlaKp is a K. pneumoniae UDP-galacturonic acid C4 epimerase providing UDP-galacturonic acid for core biosynthesis. The glaKp gene was inactivated and the structure of the mutant lipopolysaccharide was determined by mass spectrometry. The mutant displayed characteristics of a deep-rough phenotype, exhibiting a hypersensitivity to hydrophobic compounds and polymyxin B, an altered outer membrane profile, and the release of the periplasmic enzyme β-lactamase. These results indicate that the negative charge provided by the carboxyl groups of galacturonic acid do play an equivalent role to the core oligosaccharide phosphate residues in establishing outer membrane integrity in E. coli and Salmonella.

Lipopolysaccharide (LPS)† is a major virulence determinant in Gram-negative bacteria (1). The Klebsiella pneumoniae LPS molecule shares significant similarity with the well-characterized LPS structures from other members of the Enterobacteriaceae, like Escherichia coli and Salmonella (1, 2). The inner core region, which is generally well conserved in the Enterobacteriaceae, contains 3-deoxy-d-manno-oct-2-ulosonic acid (Kdo) and L-glycero-d-manno-heptopyranose (Hep), whereas the outer region shows more diversity. Conservation in the inner core may reflect its crucial role in the essential barrier function of the outer membrane (OM). There is a single core OS structure in K. pneumoniae and small variations between isolates in the addition of non-stoichiometric substituents (3). The genes responsible for core OS biosynthesis in K. pneumoniae are encoded by the waa gene cluster, whose sequence has been reported (4). The biosynthesis of the K. pneumoniae core OS region has now been well characterized, with functions having been assigned to all but one of the genes encoded in the gene cluster; the exception is orf10 (4–9). The only unassigned function is the addition of the non-stoichiometric β-galacturonic acid (GalUA) residues in the inner core (see Fig. 1), which potentially involves orf10 activity.

One major feature distinguishing the K. pneumoniae core OS from those of E. coli and Salmonella is the absence of phosphoryl substitutions (Fig. 1). The negative charges provided by these phosphate residues in E. coli and Salmonella play a critical role in maintaining the barrier function of the OM by providing sites for cross-linking of adjacent LPS molecules with divalent cations or polyamines (reviewed in Refs. 1, 10, and 11). In addition, the negative charges are important in mediating interactions between LPS and the positive charges on certain OM proteins (OMP) (12, 13). Mutants with highly truncated core OS structures lacking the inner core Hep region display a pleiotropic phenotype known as the deep-rough phenotype, characterized by changes in structure and composition of the OM (reviewed in Refs. 10, 11, 14, and 15). In E. coli and Salmonella, these mutants show a decrease in the amount of OMPs and a corresponding increase in phospholipids. The loss of OMPs is likely because of improper folding and assembly of OMPs in the absence of LPS negative charge (12, 16–22). These mutants are also hypersensitive to hydrophobic compounds, because of the appearance of phospholipids in the outer leaflet of the OM that may facilitate rapid penetration of these compounds through the phospholipid bilayer regions of the membrane. Other characteristics of deep-rough mutants seen in E. coli include the release of periplasmic enzymes, the loss of cell surface organelles (e.g. pili and flagella), secretion of an inactive form of hemolysin, and increased susceptibility to polymorphonuclear leukocyte lysosomal fractions and phagocytosis by macrophages (reviewed in Refs. 1, 10, 11, 14, and 15). In addition, there is an up-regulation of colanic acid production because of induction of the RcsC/YojN/RcsB phosphorelay system (14, 23). The RcsC/YojN/RcsB activating signal is unknown, but it is responsive to OM perturbations (24–27). One

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difficulty in relating some of the elements of the deep-rough phenotype to the LPS structure is the fact that the original findings were based on severely truncated LPS molecules with unknown genetic defects, rather than precise mutations.

Precise mutations that eliminate core phosphorylation in E. coli and Salmonella enterica sv. Typhimurium yield strains that exhibit the major characteristics of the deep-rough phenotype with an increase in susceptibility to hydrophobic compounds, but there is no alteration in the profile of the major OMPs (28, 29). The S. enterica sv. Typhimurium mutant also caused a complete attenuation of virulence in a mouse model (29).

The only source of negative charge outside the lipid A domain of K. pneumoniae is contributed by the GalUA residues in the core OS region. This is also seen in the core OS regions of other environmental isolates, Rhizobium etli (30), Rhizobium leguminosarum (30), and Plesiomonas shigelloides O54 (31). It has been proposed that having GalUA residues instead of phosphoryl substitutions may give these organisms an ecological advantage in habitats that are low in phosphate and low in the divalent cations involved in cross-linking adjacent LPS molecules, because carboxyl groups become more easily protonated, decreasing the repulsion between LPS molecules (11). To determine the role that the carboxyl groups on GalUA residues play in OM stability and whether they fulfill the same functions as the negative charges provided by the phosphate residues in E. coli and Salmonella, a mutant was created that lacked all GalUA residues from the core OS (the GalUA in the inner core as well as the non-stoichiometric substitutions in the inner core). The transferase (WabG) involved in adding the GalUA onto HepII of the inner core has been identified (7). A wabG mutant showed enhanced sensitivity to hydrophobic compounds. However, this mutant may still possess some GalUA in its inner core, because the transferase(s) responsible for the addition of the non-stoichiometric substitutions onto the HepIII and β-Glc of the inner core is presently unknown. Therefore, to entirely eliminate GalUA from the K. pneumoniae core OS, UDP-GalUA precursor synthesis was targeted. UDP-GalUA is formed from UDP-GlcUA by the UDP-GalUA C4-epimerase (formerly uge but henceforth referred to as Gla) (32, 33). A mutant in glaKP was constructed to ascertain the effect of this mutation on the OM stability of K. pneumoniae.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Growth Conditions**—The bacterial strains and plasmids used in this study are summarized in Table I. Bacteria were grown at 37 °C in Luria-Bertani broth (34). Growth media were supplemented with chloramphenicol (15 or 7.5 μg/ml), gentamicin (30 μg/ml), kanamycin (25 μg/ml), or streptomycin (200 μg/ml), as necessary. For mutant complementation, wild-type copies of glaKP were expressed using a pBAD-vector derivative, which is a family of expression vectors that use the arabinose-inducible and glucose-repressible araC promoter (35). For induction, a culture was grown at 37 °C for 18 h in LB supplemented with the appropriate antibiotics and 0.4% (w/v) glucose. This culture was diluted 1:100 into fresh medium without glucose and grown until the culture reached an 600 of 0.2. L-Arabinose, 0.02% (w/v), was then added and the culture was grown for another 2 h. Repressed controls were diluted 1:100 into fresh medium with 0.4% (w/v) glucose.

**DNA Methods**—Plasmid DNA was isolated using the Sigma GenElute Plasmid Miniprep Kit (Sigma) and chromosomal DNA was prepared by the method of Hull et al. (36), or by using DNAzol reagent (Invitrogen) in a modified protocol for bacteria (6). PCR were performed in 0.05-ml volumes with either PwoI DNA polymerase (Roche) or Platinum Taq DNA polymerase (Invitrogen), using conditions optimized for

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**FIG. 1.** The core OS structure of K. pneumoniae and E. coli K-12. The K. pneumoniae core OS structure (3, 5) is shown in panel A. Dotted arrows indicate non-stoichiometric substitutions (residues J, K, and P) with β-GalUA and Hep residues and the various combinations detected in structural analyses are given below the structure (3). Dotted lines designate the known or predicted gene products involved in the indicated linkages. The core OS structure of E. coli K-12 (2) is shown in panel B. The dotted arrows indicate non-stoichiometric substitutions.
the primer pair. Oligonucleotide primer synthesis and automated DNA sequencing were performed at the Guelph Molecular Supercenter (University of Guelph, Ontario, Canada). All PCR products were sequenced to verify that they were error-free. Plasmids were maintained in E. coli DH5α, except for pRE112 derivatives, which were maintained in DH5α [pir]. For E. coli strains, transformation was carried out by electroporation and methods described elsewhere (37). For some K. pneumoniae strains, a modification of this method was required (6, 38).

**Insertion Mutagenesis**—The mutation glaKP (CWG630) was constructed by insertion of the pRE112 plasmid into the glaKP gene on the K. pneumoniae chromosome, using methods described previously (39). Briefly, a pRE112-derivative containing a glaKP internal fragment was transformed into E. coli SM10 [pir] and then transferred by conjugation to the recipient strain, K. pneumoniae CWK2. The plasmid pRE112 requires the pir gene product to replicate (40), so for the plasmid to be maintained in CWK2, it must be integrated into the chromosome by homologous recombination within glaKP. CWK2 mutant derivatives in which pRE112 has been inserted into glaKP were selected by resistance to streptomycin (resistance carried by CWK2) and chloramphenicol (antibiotic marker on the plasmid).

An internal fragment of glaKP was amplified from K. pneumoniae CWK2 chromosomal DNA using primers KPwbnF8 (5'-AGTTGACAGCCGCCCATATC-3') and KPwbnF9 (5'-GGCCATAATTTGTCGGAGATC-3') and the blunt ends of the 510-bp product were ligated to pRE112 digested with Smal, giving pWQ68. The resulting glaKP mutant was designated K. pneumoniae CWG630. The colony morphology of the mutant was indistinguishable from the parent.

**Polyacrylamide Gel Electrophoresis Analysis**—For PAGE analysis, LPS was isolated on a small scale from protease K-digested whole cell lysates as described by Hitchcock and Brown (41). The LPS was then separated on 4–12% BisTris NuPAGE gels from Invitrogen, and visualized by silver staining (42). For SDS-PAGE of proteins, the protein samples were solubilized in SDS-containing sample buffer (43) by boiling at 100 °C for 15 min and then separated on 12% SDS-PAGE gels. Proteins were visualized by Coomassie Brilliant Blue staining.

**Determination of the Structure of Deacylated LPS**—K. pneumoniae CWG630 (glaKP) and the complemented strain CWG630 (pWQ68; His6-GlaKP) were grown in a fermenter (2×10 liters) in LB for 21 h, harvested, and then lyophilized. For the CWG630 (glaKP) rough strain lacking O-Ps (rough LPS), the LPS was isolated from the dried cells by the phenol/chloroform/light petroleum method (44). For the complemented strain producing smooth LPS, CWG630 (pWQ68; GlacK), the hot water phenol method of LPS extraction was used (45). LPS was deacylated by procedures described elsewhere (46, 47). Briefly for O-deacylation, the LPS (100 mg) was dissolved in anhydrous hydrazine (3 ml), incubated for 1 h at 40 °C, and then poured into cold acetone. The precipitated O-deacylated LPS was collected by centrifugation, washed with acetone, and then lyophilized. Electrospray ionization (ESI) mass spectra were obtained using a Micromass Quattro spectrometer (VG Instruments Inc., Hudson, NH) in 50% MeCN with 0.2% HCOOH, at a flow rate of 15 μl/min with direct injection.

**OM Protein Preparations**—For OM preparations, bacteria were grown overnight at 37 °C. The culture was then diluted 1:100 in 100 ml of fresh medium and incubation was continued at 37 °C for 18 h. The cells were harvested and frozen until required. The cell pellet was thawed, resuspended in 10 ml of 10 mM Tris, pH 8.0, and sonicated on ice (for a total of 2 min using 10-s bursts, followed by 10-s cooling periods). Unbroken cells and cell debris was removed by centrifugation (10 min at 20,000 × g). The membrane fraction was isolated by ultracentrifugation (60 min at 100,000 × g). The membrane fraction was resuspended in 1 ml of 10 mM Tris, pH 8.0, containing 2% Sarkosyl (sodium lauryl sarcosine) and incubated on a rotary shaker at room temperature for 30 min. The OM is resistant to Sarkosyl solubilization, whereas the inner membrane is not (45). The Sarkosyl-insoluble OM was separated from the soluble inner membrane by centrifugation at 100,000 × g for 15 min. The OM fraction was washed again with 2% Sarkosyl, washed twice with 10 mM Tris, pH 8.0, and then resuspended in 0.2 ml of 10 mM Tris, pH 8.0. The protein content of each sample was determined using the Bio-Rad DC protein assay, according to the manufacturer’s instructions. Unlike E. coli, the inner and outer membranes of these K. pneumoniae strains could not be reliably separated by isopycnic sucrose gradient centrifugation. The molecular basis for this is unclear, but it is not a unique situation (49).

Identification of individual OM proteins was performed at the Biological Mass Spectrometry Facility, University of Guelph, by peptide mass fingerprinting and matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) analysis. Data base searching was done using the ProFound search engine (prowl.rockefeller.edu/).

**Sensitivity to Hydrophobic Compounds and Polymyxin B (PMB)**—For SDS sensitivity, serial 2-fold dilutions of SDS (from 6.25 to 0.098 mg/ml) were made in 5 ml of LB. An 18-h culture of each strain was used to inoculate each 5-ml tube at a ratio of 1:100. After incubation for 8 h at 37 °C, a tube was rated positive for growth if the optical density at 600 nm was greater than 0.2. For sensitivity to novobiocin, vancomycin, and PMB, a plate assay was performed. An 18-h culture grown at 37 °C was added to an LB plate supplemented with antibiotics, where appropriate, and the excess was poured off. The plates were dried and 0.01 ml of serial 2-fold diluted novobiocin (from 51.2 to 0.2 mg/ml), vancomycin (from 40 to 0.0098 mg/ml), or PMB (from 780 to 0.095 μg/ml) were spotted onto the plates. After a 24-h incubation at 37 °C, the minimum inhibitory concentration (MIC) was determined as the highest dilution resulting in clearing. All assays were performed in triplicate.

**Spectrophotometric Assay for Detection of β-Lactamase Activity**—To measure endogenous β-lactamase activity, bacteria were grown at 37 °C for 18 h in LB supplemented with the appropriate antibiotics. The culture was then diluted 1:50 in 200 ml of fresh medium and incubation was continued at 37 °C for 4 h. 1 ml of cells were pelleted and the
culture supernatant was retained and kept on ice. The remaining cells were harvested and the cell pellet was resuspended in 5 ml of cold LB. The cells were lysed by sonication and the unbroken cells and cell debris were removed by low speed centrifugation (15 min at 5000 × g). The cell lysate was diluted to 10 times the original culture volume, because of the high levels of β-lactamase activity. 160 μl of supernatant and diluted cell lysate from each strain was aliquoted in triplicate into 96-well polystyrene microplates (Evergreen Scientific Inc., Los Angeles, CA). Reactions were started by the addition of 40 μl of a 0.5 mg/ml nitrocephin stock (0.1 mg/ml final concentration; Oxoid, Basingstoke, UK) and the absorbance at 560 nm was measured every minute for 30 min using a FLUOstar OPTIMA microplate reader (BMG Labtechnologies Inc., Offenburg, Germany). A sample of 160 μl of LB growth medium with 40 μl of nitrocephin was used to measure background absorbance. Each strain was assayed in triplicate in multiple experiments.

RESULTS

GlaKP Is Required for GalUA Addition to the Core—To examine the role of glaKP in core OS biosynthesis, a gene disruption mutant K. pneumoniae (CWG630) was constructed. The lipid A-core fraction of the LPS of the glaKP mutant migrated significantly faster than wild-type CWK2 LPS on PAGE, indicative of the loss of several residues from the core (Fig. 2, lane 2). In addition, the glaKP mutant lacked the high molecular weight LPS species, consistent with the loss of the known O-PS ligation site in the outer core region (5). Wild-type core OS migration and O-PS ligation were restored when pWQ69 (His6-GlaKP) was introduced into the mutant strain (Fig. 2, lane 3).

The structure of the CWG630 (glaKP) mutant LPS was determined by ESI-MS after O-deacylation with hydrazine (Fig. 3B). The spectrum shows 4 main peaks that are all derivatives of the same structure (structure 5). Each lacks all GalUA residues from the core, as well as the HepIII residue. This produces a truncated core OS, because GalUA represents the first residue in the outer core (compare with structures of the complete core OS of CWG399 (waaL) in Fig. 3A). The branching HepIII residue in the inner core is also missing in structural analysis of other mutants lacking the first residue in the outer core, including a K. pneumoniae waaG mutant that adds GalUA onto HepII (7) and an E. coli F470 mutation in waaG that eliminates the transferase responsible for adding the first Glc residue to the outer core OS in E. coli (see Fig. 1B). The glaKP mutant (CWG630) showed peaks at m/z 2071.0 and 2094.0 with corresponding structures showing 4-amino-4-deoxyarabinose (Ara4N) substitutions on the lipid A moiety.

The structure of the O-deacylated LPS of the complemented strain CWG630 (pWQ69; His6-GlaKP) showed the same four peaks as those in CWG399 (waaL) (peaks with m/z 2690.0, 2866.0, 2882.0, and 3058 corresponding to structures 1, 2, 3, and 4, respectively), as well as two peaks at m/z 2998.0 and 3129.0 corresponding to structure 3 containing Ara4N substitutions (Fig. 3C). Each identified peak in the complemented mutant LPS is accompanied by a corresponding peak +38 mass units. The source of the additional peaks is unknown, but they might represent the effect of overexpressing a single gene relative to the rest of the system. Interestingly, when the LPS from the wild-type CWK2 strain was analyzed there were no peaks identified that could correspond to structures bearing Ara4N substitutions (3). Ara4N substitutions remain in CWG630 (pWQ69; His6-GlaKP). There are two possible explanations. It is possible that the full spectrum of changes induced by the glaKP defect could not be reversed by the complementing plasmid. Alternatively, the appearance of Ara4N may reflect a secondary compensatory mutation arising to accommodate the glaKP defect.

Sensitivity of CWG630 (glaKP) to Hydrophobic Compounds and PMB—The OM of Gram-negative bacteria is an effective permeability barrier, allowing only limited diffusion of hydrophobic compounds. However, mutants with a defective OM exhibiting a deep-rough phenotype typically show hypersensitivity toward hydrophobic compounds, anionic or neutral detergents, and cationic peptides (reviewed in Refs. 1, 10, 11, 15, and 50). Vancomycin is a glycopeptide antibiotic that has a narrow spectrum of activity limited to Gram-positive bacteria (51). Gram-negative bacteria are usually not susceptible to vancomycin, because the large polar molecule is unable to penetrate an intact OM (51). To determine whether CWG630 (glaKP) exhibits altered OM properties, the sensitivity of the mutant was tested against the hydrophobic antibiotic novobiocin, the glycopeptide vancomycin, the anionic detergent SDS, and the cationic peptide PMB (Table II). CWG630 (glaKP) showed a 16-fold increase in sensitivity to SDS, novobiocin, and PMB, in comparison to the wild-type K. pneumoniae CWK2 and a 51.3-fold increase in sensitivity to vancomycin. Complementation of CWG630 (glaKP) with His6-GlaKP on a plasmid (pWQ69) restored the MIC to wild-type for vancomycin, near wild-type for PMB and resulted in an MIC for SDS and novobiocin that exceeded wild-type levels. The latter results probably reflect the expression of His6-GlaKP from multicopy expression plasmids at levels exceeding chromosomal copy and the resulting increase in UDP-GalUA pools available in the cell. However, the interpretation is complicated by residual Ara4N in the LPS of the complemented mutant. Two additional controls were used: the K. pneumoniae mutant CWG399 (waaL) that lacks O-PS, but otherwise has a full-length core OS, and the CWG603 (waaL wabH) mutant that has the same core OS structure as CWG630 (glaKP) except for the presence of GalUA residues and HepIII (6). CWG399 (waaL) and CWG603 (waaL wabH) showed a 4-fold decrease in MIC values for SDS and PMB, and a 2-fold decrease for vancomycin, which may be attributed to the fact that these two mutants lack O-PS, in comparison to wild-type K. pneumoniae. No change in sensitivity to novobiocin was seen.

OM Profile of CWG630 (glaKP)—To examine the effect of the glaKP mutation on OM structure, the OMPs were prepared from CWK2 and CWG630 (glaKP) (Fig. 4A) and analyzed by SDS-PAGE. There was no difference in the spectrum of protein bands seen in the two strains, but there was a change in the relative intensities of some of the protein bands (Fig. 4A). In the glaKP mutant, Bands 1 and 2 increased in amount in the mutant, whereas there was a decrease in the amount of Bands...
Fig. 3. Structural analysis of the LPS from CWG630 (glaKP). Panel A shows the ESI-MS mass spectrum obtained from O-deacylated LPS of *K. pneumoniae* CWG399 (waaL) and the corresponding structures of the main peaks (as reported previously (5)). Non-stoichiometric substitutions in the CWG399 (waaL) LPS are represented by residues J and P. The β-GalUA substitution (residue K) on HepIII in Fig. 1 was not present, because it is only added if a β-GalUA residue is first attached onto the β-Glc residue (residue K) (E. Vinogradov, unpublished results). The ESI-MS mass spectra and the structural assignments of the main peaks in the spectra from the O-deacylated LPS of CWG630 (gla) and CWG630 (pWQ69; His6-GlaKP) are shown in panels B and C, respectively. The structure shown in panel B corresponds to structure 5 identified in the mass spectrum. Lipid A* in these structures consists of a β-GlcN(acyl)4P-(1,6)-α-GlcN(acyl)1P backbone, where acyl represents a 3-hydroxytetradecanoyl residue. A portion of the lipid A*-core OS region in CWG630 (glaKP) or CWG630 (pWQ69; His6-GlaKP) is substituted with Ara4N.
His 6-GlaKP A K. pneumoniae from other sugars with disaccharides having a larger binding by ScrY allows for the diffusion of sucrose, as well as a variety was found to be the sucrose porin (ScrY). The channel formed ophages and colicins (Ref. 54, and references therein). Band 6 in nucleoside transport, as well as serves as the transporter for in the Enterobacteriaceae, it is involved constant than disaccharides (55, 56). These two proteins form substrate-specific channels in the outer membrane but it is apparent that reduction of these proteins in the glaKP mutant does not affect the growth of K. pneumoniae in the laboratory.

The remaining proteins identified in the OMP profiles of CWK2 and CWG630 (glaKP) were conserved between the two strains. Bands 4, 5, 9, and 10 were identified as previously characterized K. pneumoniae proteins. Band 4 corresponds to OmpK36, the K. pneumoniae homolog of the E. coli porin protein OmpC (57). K. pneumoniae also produces an OmpK35 porin (equivalent to E. coli OmpF) (58) that was not detected in these OMP preparations. Band 5 represents K. pneumoniae OmpA, a major OMP that is highly conserved in the Enterobacteriaceae. OmpA homologs have also been identified in a wide range of Gram-negative bacteria (59). OmpA has several functions: stabilizes the OM, acts as a slow nonspecific porin, plays a role in bacterial conjugation, and serves as a bacteriophage receptor (reviewed in Ref. 60 and 61). Bands 9 and 10 both corresponded to the same protein OmpK17. It appears to be processed to a smaller size in the glaKP mutant. OmpK17 is a K. pneumoniae 17-kDa OM protein of unknown function that is present in several K. pneumoniae serotypes (62).

**Release of β-Lactamase in the Supernatant by CWG630 (glaKP)**—Mutants with a deep-rough phenotype have also been reported to release periplasmic enzymes into the supernatant because of resulting defects in the OM (63, 64). β-Lactamase is a periplasmic enzyme in Gram-negative bacteria (51, 65) that can be encoded on the chromosome or on plasmids. Both types have been detected in K. pneumoniae, providing intrinsic resistance to both penicillins and cephalosporins (51, 65, 66). The chromogenic cephalosporin nitrocefin undergoes a color change when hydrolyzed by β-lactamasases. It was used to compare the amount of β-lactamase activity present in the cells of CWG630 (glaKP) with the amount released from the periplasm into the supernatant, to determine whether this mutant displays an increase in the release of periplasmic enzymes. The amount of β-lactamase present in the supernatant was significantly higher for CWG630 (glaKP) than for the wild-type CWK2 (Table IV). When GlαKP was added back to CWG630 (glaKP) the amount of β-lactamase in the supernatant was reduced to wild-type levels. The control strains CWG399 (waaL) and CWG603 (waaL wabH) showed near wild-type profiles, with a slight increase in β-lactamase release in CWG603 (waaL wabH).

**DISCUSSION**

The emergence of antibiotic-resistant K. pneumoniae strains, particularly extended-spectrum β-lactamase-resistant-producing isolates that are difficult to treat, has renewed research on this pathogen to develop new therapeutic strategies (67–69). The LPS of K. pneumoniae serves as an important virulence factor. It has been shown that isolates of serotypes O1 and O2 release during growth an extracellular toxic complex composed of LPS, capsule, and a small amount of protein (70, 71). Extracellular toxic com-

### Table II

| Strain                  | SDS<sup>a</sup> | Vancomycin<sup>b</sup> | Novobiocin<sup>b</sup> | Polymyxin B<sub>5</sub><sup>b</sup> |
|------------------------|-----------------|------------------------|------------------------|-------------------------------|
| K. pneumoniae CWK2     | 3.13            | 20                     | 3.2                    | 48                            |
| K. pneumoniae CWG399 (waaL) | 0.78        | 10                     | 3.2                    | 12                            |
| K. pneumoniae CWG603 (waaL wabH) | 0.78 | 10                     | 3.2                    | 12                            |
| K. pneumoniae CWG630 (glaKP) | 0.19     | 0.39                   | 0.2                    | 3.05                          |
| K. pneumoniae CWG630 (pWQ69; GlaKP<sup>−</sup>) | >6.25     | 20                     | 6.4                    | 24                            |

<sup>a</sup> MIC were determined in 5 ml of LB broth.

<sup>b</sup> MIC were determined plate assay.

**Fig. 4. OM profile of CWG630 (glaKP).** Panel A, OMPs of CWK2 and CWG630 (glaKP) were separated on a 12% SDS-PAGE gel. Proteins indicated by Bands 1 and 2 were present in higher amounts in the mutant CWG630 (glaKP), whereas those represented by Bands 3 and 6 were present in lower amounts. Band 7 is very faint on the gel in panel A, but can be seen in B. Panel B, OMPs of the control strains CWG630 (pWQ69; His<sub>6</sub>-GlaKP<sup>−</sup>), CWG399 (waaL), and CWG603 (waaL wabH) were separated on a 12% SDS-PAGE gel. A total of 10 μg of each OMP preparation was loaded to standardize SDS-PAGE analysis.

3 and 6. OMPs from the complemented strain CWG630 (glaKP) (pWQ69; His<sub>6</sub>-GlaKP<sup>−</sup>) and the control strains CWG399 (waaL) and CWG603 (waaL wabH) showed wild-type profiles (Fig. 4B), indicating that the changes resulted from the glaKP mutation, rather than being a consequence of general LPS mutations. Ten proteins (Bands 1–10) in the OMP profile were identified by peptide mass fingerprinting and MALDI-MS analysis and the results are shown in Table III. The inability to definitively identify Bands 1, 7, and 8 reflects the limited number of K. pneumoniae proteins available in the data base, as the K. pneumoniae genome sequence is not yet complete.

Band 2 showed similarity to a putative OM antigen YaET of S. enterica sv. Typhimurium. YaET is a homolog of the Omp85 protein of Neisseria meningitidis. Omp85 is essential to cell viability and is involved in OMP assembly (52, 53). The relative amount of Band 2 was higher in the mutant.

Band 3 was identified as the nucleoside-specific Tsx channel from K. pneumoniae. In the Enterobacteriaceae, it is involved in nucleoside transport, as well as serves as the transporter for the antibiotic albicin and as the receptor for several bacteriophages and colicins (Ref. 54, and references therein). Band 6 was found to be the sucrose porin (ScrY). The channel formed by ScrY allows for the diffusion of sucrose, as well as a variety of other sugars with disaccharides having a larger binding constant than disaccharides (55, 56). These two proteins form substrate-specific channels in the outer membrane but it is apparent that reduction of these proteins in the glaKP mutant does not affect the growth of K. pneumoniae in the laboratory.
plex is associated with the extensive lung damage and high lethality typically seen in mice with \textit{Klebsiella}-derived pneumonia (70, 71). This toxicity is mainly because of the presence of LPS molecules with divalent cations. In the absence of GalUA, the addition of Ara4N onto the phosphate groups on the lipid A in the mutant would potentially decrease the electrostatic repulsion between the phosphate groups on neighboring LPS molecules and may reflect an attempt by the mutant to stabilize the OM.

The \textit{glaKP} mutant (CWG630) showed evidence of significant perturbations in its permeability barrier function. In comparison to the wild-type strain, a mutant lacking O-PS, and a mutant in the \textit{wabH} gene product (CWG603) whose LPS lacks all residues in the outer core beyond GalUA (Fig. 1), CWG630 (\textit{glaKP}), shows an altered OM profile, hypersensitivity to hydrophobic compounds, the large hydrophilic glycopeptide antibiotic vancomycin, and the cationic peptide PMB, and releases significantly higher amounts of the periplasmic enzyme \textit{\beta}-lactamase. In many respects, this phenotype resembles a \textit{waaP} mutation in \textit{E. coli} (28) and in \textit{S. enterica} sv. Typhimurium (29), which eliminates all phosphoryl groups of the core OS. Therefore, the negative charge contributed by GalUA apparently plays a role in the maintenance of a stable OM in \textit{K. pneumoniae} that is equivalent to the role of the phosphate residues present in the core OS of \textit{E. coli} and \textit{Salmonella}.

The cationic peptide PMB is a cyclic heptapeptide with a fatty acid tail having a net positive charge of 5 (73, 74). The mechanism by which cationic peptides interact with bacterial membranes and the method by which they exert their bactericidal effects is complex. PMB has been proposed to act in a detergent-like mechanism, in which accumulation of PMB at the membrane surface leads to the formation of aggregates of detergent-like mechanism by which cationic peptides interact with bacterial membranes, such as the negatively charged phospholipids (phosphatidylglycerol and phosphatidic acid) and especially LPS molecules (reviewed in Ref. 74). Strong electrostatic interactions have been reported between polycationic peptides and polyamionic membrane components, such as the negatively charged phospholipids (phosphatidylglycerol and phosphatidic acid) and especially LPS molecules (reviewed in Ref. 74). The anionic groups (carboxyl groups or phosphate residues) present on LPS are important in PMB binding to LPS. Of particular importance are the carboxyl groups or phosphate residues) present on LPS that are not present in sensitive strains (75).

\begin{table}[h]
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\caption{Identification of OMPs from CWG630 (\textit{glaKP}) and the wild-type CWK2 (Fig. 4)}
\begin{tabular}{lllllll}
\hline
Band & Organism & Protein identified$^a$ & Accession number & Ref. & Probability & Estimated Z & Coverage of matched peptides & Predicted mass \\
\hline
1$^b$ & \textit{Yersinia pestis} KIM & Hypothetical protein & NP\_668086\_1 & 82 & 0.56 & 0.22 & 21 & 77.77 \\
2 & \textit{S. enterica} sv. Typhimurium & Putative outer membrane antigen & NP\_459229\_1 & 83 & 0.98 & 0.71 & 23 & 89.52 \\
3 & \textit{K. pneumoniae} & Nucleoside-specific channel forming protein Tax precursor & P40786 & 54 & 1 & 2.39 & 50 & 33.49 \\
4 & \textit{K. pneumoniae} & OmpK36 & CAC50885\_1 & 84 & 1 & 2.17 & 52 & 40.79 \\
5 & \textit{K. pneumoniae} & Outer membrane protein A precursor & JC6558 & 85 & 1 & 2.26 & 69 & 38.14 \\
6 & \textit{K. pneumoniae} & Soluteoporin precursor & P27218 & 55 & 1 & 2.38 & 62 & 55.63 \\
7$^b$ & \textit{Proteus mirabilis} & PmFD & CA84591\_1 & 86 & 0.24 & 0.11 & 39 & 28.36 \\
8$^b$ & \textit{E. coli} & MoeB & NP\_308931\_1 & 87 & 0.45 & 0.19 & 23 & 27.2 \\
9 & \textit{K. pneumoniae} & OmpK17 & AAA97932\_1 & 62 & 1 & 1.59 & 68 & 18.45 \\
10 & \textit{K. pneumoniae} & OmpK17 & AAA97932\_1 & 62 & 1 & 2.09 & 68 & 18.45 \\
\hline
\end{tabular}
\footnotesize
\textsuperscript{a} Proteins identified by MALDI-MS peptide mass fingerprinting and database searching using the ProFound search engine. Searches were restricted to the Enterobacteriaceae because of the limited number of proteins in the database for \textit{K. pneumoniae}. For Band 7, the search was restricted to 0–31-kDa proteins, in an attempt to improve identifications.
\textsuperscript{b} Results are unreliable.
\end{table}
and reduce the surface charge density to decrease PMB binding. The location of the negative charges and the neutralizing substitutions are important in determining the degree of resistance (76). Some data also implicates hydrophobic interactions in the binding of PMB to LPS (77), highlighting the complexity of these interactions. Even though the net negative charge in the core OS of CWG630 (\(g\alphaKP\)) has decreased because of the lack of GalUA residues and the presence of Ara4N substitutions, the strain showed increased sensitivity to PMB. A similar result was seen with a \(waaC\) mutation in \(S.\ enterea\) sv. Typhimurium that results in a loss of core OS phosphorylation (29). This mutant showed increased sensitivity to PMB, even though it showed normal substitution with Ara4N. This may indicate that PMB binding to the OM in deep-rough mutants by means of hydrophobic interactions may outweigh the benefits gained by a decrease in net negative charge (29). However, a definitive explanation awaits determination of the precise mechanism of PMB action.

It has been demonstrated that the negative charges in the inner core OS are involved in the assembly and insertion of OM proteins (19, 21), explaining the decrease in OMPs seen in deep-rough mutants. The OM profile of the \(g\alphaKP\) mutant (CWG630) did show a decrease in the amount of two substrate-specific OM channels, the nucleoside-specific Tss channel and the sucrose porin ScrY. The OM of deep-rough mutants has been shown to contain phospholipid bilayer regions (78), possibly as a result of phospholipids filling in the gaps in the OM resulting from the loss of OMPs. In this respect, it is interesting that the \(g\alphaKP\) mutant (CWG630) showed an increase (relative to wild-type) in a homolog of \(YaeT\) of \(K.\ pneumoniae\) OM (28, 29).

Many biological phenomena are studied extensively in \(E.\ coli\) and \(Salmonella\) and it is assumed that other members of the Enterobacteriaceae will share a high degree of similarity. It is worth emphasizing the importance of examining other members of this family, as important differences do exist. The results presented indicate that \(K.\ pneumoniae\), like \(E.\ coli\) and \(Salmonella\), utilize core OS negative charges to maintain OM integrity. However, unlike \(E.\ coli\) and \(Salmonella\), the core OS negative charge in \(K.\ pneumoniae\) is provided by GalUA residues and not phosphate residues. This potentially reflects in the habitats of these organisms and the availability of phosphates in these habitats.

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