Characterization of Gla_{KP}, a UDP-Galacturonic Acid C4-Epimerase from *Klebsiella pneumoniae* with Extended Substrate Specificity

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In *Escherichia coli* and *Salmonella enterica*, the core oligosaccharide backbone of the lipopolysaccharide is modified by phosphoryl groups. The negative charges provided by these residues are important in maintaining the barrier function of the outer membrane. In contrast, *Klebsiella pneumoniae* lacks phosphoryl groups in its core oligosaccharide but instead contains galacturonic acid residues that are proposed to serve a similar function in outer membrane stability. Gla_{KP} is a UDP-galacturonic acid C4-epimerase that provides UDP-galacturonic acid for core synthesis, and the enzyme was biochemically characterized because of its potentially important role in outer membrane stability. High-performance anion-exchange chromatography was used to demonstrate the UDP-galacturonic acid C4-epimerase activity of Gla_{KP}, and capillary electrophoresis was used for activity assays. The reaction equilibrium favors UDP-galacturonic acid over UDP-glucuronic acid in a ratio of 1.4:1, with the K_m for UDP-glucuronic acid of 13.0 μ M. Gla_{KP} exists as a dimer in its native form. NAD⁺/NADH is tightly bound by the enzyme and addition of supplementary NAD⁺ is not required for activity of the purified enzyme. Divalent cations have an unexpected inhibitory effect on enzyme activity. Gla_{KP} was found to have a broad substrate specificity in vitro; it is capable of interconverting UDP-glucose/UDP-galactose and UDP-N-acetylglucosamine/UDP-N-acetylgalactosamine, albeit at much lower activity. The epimerase GalE interconverts UDP-glucose/UDP-galactose. Multicopy plasmid-encoded *gla*_{KP} partially complemented a *galE* mutation in *S. enterica* and in *K. pneumoniae*; however, chromosomal *gla*_{KP} could not substitute for *galE* in a *K. pneumoniae* *galE* mutant in vivo.

Galacturonic acid (GalUA) is a major constituent of bacterial polysaccharides and plant cell walls. In bacteria, it can be found in capsule structures and as part of the lipopolysaccharide (LPS) molecule in several different bacterial species. LPS is a major virulence determinant in gram-negative bacteria (50). In the *Enterobacteriaceae*, the LPS molecule can be subdivided into three regions: (i) lipid A, the hydrophobic membrane anchor; (ii) a core oligosaccharide (core OS); and (iii) a polymer of glycosyl (repeat) units known as O polysaccharide (O-PS). Depending on the bacterial species, GalUA is found as a substituent on the β -(1, 6)-linked GlcN disaccharide backbone of lipid A (73) and as a monosaccharide component of the core OS region (21) and the O-PS (28).

The *Klebsiella pneumoniae* LPS molecule (Fig. 1) shares significant similarity with the well-characterized LPS structures from other members of the *Enterobacteriaceae*, like *Escherichia coli* and *Salmonella enterica* (21, 50). However, one major feature distinguishing the *K. pneumoniae* core OS from that of *E. coli* and *S. enterica* is the absence of phosphoryl substitutions (Fig. 1). The negative charges provided by these phosphate residues in *E. coli* and *S. enterica* play an important role in maintaining the barrier function of the outer membrane (OM) by providing sites for divalent cations to cross-link adjacent LPS molecules (reviewed in reference 50). Mutants with highly truncated core OS structures lacking the inner core heptose-containing region display a pleiotrophic phenotype known as the deep-rough phenotype, characterized by changes in struc-

ture and composition of the OM (reviewed in references 18, 41, 42, and 56). In *E. coli* and *Salmonella*, these mutants show a decrease in the amount of OM proteins and a corresponding increase in phospholipids. These mutants are also hypersensitive to hydrophobic compounds, due to the appearance of phospholipids in the outer leaflet of the OM, which 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 the upregulation of colanic acid production (reviewed in reference 50). Precise mutations that eliminate core phosphorylation in *E. coli* and *S. enterica* serovar Typhimurium yield strains that exhibit some of the major characteristics of the deep-rough phenotype. They are characterized by an increase in susceptibility to hydrophobic compounds, but there is no alteration in OM protein profile (71, 72). The *S. enterica* serovar Typhimurium mutant also caused a complete attenuation of virulence in a mouse model (71).

The core OS of *K. pneumoniae* contains GalUA residues as the only source of negative charge outside the lipid A-Kdo inner core domain. This is also the case in the core OS regions of *Rhizobium etli* and *Rhizobium leguminosarum* (14) and of *Plesiomonas shigelloides* O54 (40). In addition, some organisms have GalUA replacing the phosphate residues present on the lipid A moiety, such as *R. etli* (14), *R. leguminosarum* (6), and *Aquifex pyrophilus* (48). Interestingly, all these bacteria are environmental isolates, as is the case with *K. pneumoniae* (2). 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

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FIG. 1. The core OS structure of *K. pneumoniae* and *E. coli* K-12. The *K. pneumoniae* core OS structure is shown in panel A. Dashed arrows indicate nonstoichiometric substitutions. In *K. pneumoniae* these substitutions (residues J, K, and P) are comprised of β -GalUA and Hep residues, and the various combinations detected in structural analyses are given below the structure (66, 67). The core OS structure of *E. coli* K-12 (21) is shown in panel B.

the divalent cations involved in cross-linking adjacent LPS molecules, since carboxyl groups become more easily protonated, decreasing the repulsion between LPS molecules (41). Interestingly, the carboxyl groups on GalUA residues in homogalacturonan polymers (a component of pectic polysaccharides) in plant cell walls are cross-linked to each other by Ca^{2+} ions, contributing to the structural integrity of plant cell walls (reviewed in reference 43). Studies of mutants with LPS defects indicate that the carboxyl groups of the GalUA sugars in the core OS of *K. pneumoniae* provide the negative charge needed for OM stability (52; E. Fridrich, E. Vinogradov, and C. Whitfield, unpublished results).

Unlike other bacteria whose lipid A or inner core contains GalUA residues, *K. pneumoniae* is also an opportunistic pathogen. It is implicated in severe diseases, including urinary tract infections, pneumonia, and bacteremia, that are normally hospital acquired (reviewed in reference 49). The emergence of antibiotic-resistant *K. pneumoniae* strains, particularly extended-spectrum β -lactamase-producing isolates that are difficult to treat, has renewed research on this pathogen in order to develop new therapeutic strategies (49). Since GalUA residues play a significant role in the biology of the LPS molecule of *K. pneumoniae* due to its importance in the maintenance of OM stability, the UDP-GalUA C4-epimerase involved in UDP-GalUA precursor synthesis was biochemically characterized.

UDP-GalUA is synthesized from UDP-Glc. UDP-Glc is first converted to UDP-glucuronic acid (GlcUA) by the UDP-glucose dehydrogenase (Ugd), converting the primary alcohol

group to the corresponding acid. UDP-GlcUA is then converted to UDP-GalUA by the UDP-GalUA C4-epimerase (henceforth referred to as Gla). The *K. pneumoniae* UDP-GalUA C4-epimerase was originally named *uge* by another group of investigators (52). However, bacterial UDP-GalUA C4-epimerases were given the gene designation *gla* by the bacterial polysaccharide gene nomenclature system (<http://www.microbio.usyd.edu.au/BPGD/default.htm>) (51, 55), and Gla was adopted in this report with a subscript designating the organism, i.e., Gla_{KP} for the gene from *K. pneumoniae*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are summarized in Table 1. Bacteria were grown at 37°C in Luria-Bertani (LB) broth or in M9 minimal medium supplemented with 0.4% (wt/vol) glucose (33). Growth media were supplemented with chloramphenicol (15 µg/ml or 7.5 µg/ml), kanamycin (25 µg/ml), or streptomycin (200 µg/ml), as necessary. For mutant complementation, wild-type copies of *glgK_P* and *galE* were expressed using pBAD-vector derivatives in the relevant mutant strains. Plasmid pBAD18-Km belongs to a family of expression vectors that use the arabinose-inducible and glucose-repressible *araC* promoter (17). For induction, a culture was grown at 37°C for 18 h in LB supplemented with either chloramphenicol or kanamycin and 0.4% (wt/vol) glucose. This culture was diluted 1:100 into fresh medium without glucose and grown until the culture reached an optical density at 600 nm of 0.2. 0.02% (wt/vol); L-arabinose was then added, and the culture was grown for another 2 h. Repressed controls were diluted 1:100 into fresh medium with 0.4% (wt/vol) glucose. For complementation studies using strains grown in M9 minimal medium containing 0.4% (wt/vol) glucose, induction was carried out with 0.6% (wt/vol) L-arabinose.

DNA methods. Plasmid DNA was isolated using the Sigma GenElute Plasmid Miniprep Kit, and chromosomal DNA was prepared by the method of Hull et al. (22) or by using DNAzol Reagent (Invitrogen) in a modified protocol for bacteria (16). PCRs were performed in 0.05-ml volumes with either *PwoI* DNA polymerase (Roche) or Platinum *Taq* DNA polymerase (Invitrogen), using conditions optimized for the primer pair. Oligonucleotide primer synthesis and automated DNA sequencing were performed at the Guelph Molecular Supercentre (University of Guelph, Ontario, Canada). All PCR products were sequenced to verify that they were error free. Plasmids were maintained in *E. coli* DH5 α , except for pRE112-derivatives that were maintained in DH5 α [λ *pir*]. For *E. coli* strains, transformation was carried out by electroporation and by methods described elsewhere (7). For some *K. pneumoniae* strains, a modification of the electroporation method was required (10, 16).

Insertion mutagenesis. The mutation in *galE* (CWG631) was constructed by insertion of the pRE112 plasmid into the *galE* gene on the *K. pneumoniae* chromosome, using methods described previously (39). Briefly, a pRE112-derivative containing a *galE* internal fragment was transformed into *E. coli* SM10[λ *pir*] and then transferred by conjugation to the recipient strain, *K. pneumoniae* CWK2. The plasmid pRE112 requires the *pir* gene product to replicate (34), so in order for the plasmid to be maintained in CWK2, it must be integrated into the chromosome by homologous recombination within *galE*. CWK2 mutant derivatives in which pRE112 has been inserted into *galE* were selected by resistance to streptomycin (resistance carried by CWK2) and chloramphenicol (antibiotic marker on the plasmid).

The internal *galE* fragment was PCR amplified from *K. pneumoniae* CWK2 chromosomal DNA using primers KpWaa50 (5'-GCACCGCGTACCATCTT C-3') and KpWaa51 (5'-GAGTCGTCGCCAAGCCGT C-3'). The 431-bp PCR product was digested with KpnI (site underlined) and ligated to pRE112 digested with SmaI and KpnI to form pWQ73. The *galE* mutant was designated *K. pneumoniae* CWG631.

Plasmid constructs for GalE and Gla expression. The *gla_{KP}* gene was amplified from *K. pneumoniae* CWK2 chromosomal DNA using primers KPwbnF13 (5'-cccaagcTTAGATCTGATAGTAATCCTTG-3') (lowercase letters indicate changes from the original genome sequence) and KPwbnF14 (5'-ggaattccatATGAAGTTTTGGTCACTGG-3'). The 1,023-bp PCR product was digested by using HindIII and NdeI (sites underlined) and ligated into the same sites in pET28a(+) to make plasmid pWQ67. This plasmid expresses Gla_{KP} with an in-frame six-histidine (His₆) tag fused to the N terminus. For complementation studies, pWQ69 was constructed, in which the insert from pWQ67 was cloned in pBAD18-Km to facilitate expression in *K. pneumoniae*. The *galE* gene from *K. pneumoniae* was PCR amplified from CWK2 chromosomal DNA with primers

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype, serotype, or description	Reference or source
<i>E. coli</i>		
BL21[λ DE3]	F ⁻ <i>ompT hsdS_B(r_B⁻m_B⁻) gal dcm</i> [λ DE3]	Novagen
DH5 α	K-12 ϕ 80d <i>deoR lacZΔM15 endA1 recA1 hsdR17(r_K⁻m_K⁺) supE44 thi-1 gyrA96 relA1 Δ(lacZYA-argF)U169 F⁻</i>	54
DH5 α [λ pir]	K-12 ϕ 80d <i>deoR lacZΔM15 endA1 recA1 hsdR17(r_K⁻m_K⁺) supE44 thi-1 gyrA96 relA1 Δ(lacZYA-argF)U169 F⁻ [λ pir]</i>	9
SM10[λ pir]	K-12 <i>thi-1 thr-1 leuB6 tonA21 lacY1 supE44 glnV44 recA::RP4-2-Tc::Mu</i> [λ pir]	34
<i>S. enterica</i> Serovar Typhimurium	<i>galE503</i> ; R-LPS	SGSC163 ^a
LT2 SL1306		
<i>K. pneumoniae</i>		
CWK2	O1:K ⁻ ; derivative of CWK1 (O1:K20); Str ^r Ap ^r	68
CWG631	<i>galE::pRE112</i> derivative of CWK2; Cm ^r	This study
Plasmids		
pBAD18-Km	Arabinose-inducible expression vector; Km ^r	17
pET28a(+)	IPTG-inducible expression vector; Km ^r	Novagen
pRE112	Mobilizable suicide vector used for chromosomal insertions requiring λ pir for replication; Cm ^r	9
pWQ67	pET28a(+)-derivative expressing Gl _{KP} with an N-terminal His ₆ -tag; Km ^r	This study
pWQ69	pBAD18-Km-derivative expressing His ₆ -Gl _{KP} ; Km ^r	This study
pWQ72	pBAD18-Km-derivative expressing GalE; Km ^r	This study
pWQ73	pRE112-derivative containing an internal fragment of <i>galE</i> , used to construct CWG631; Cm ^r	This study

^a *Salmonella* Genetic Stock Center.

KPwaa48 (5'-GACGATGTGcATGcTCAACGGGGT-3'; (lowercase letters indicate changes from the original genome sequence) and KPwaa49 (5'-CTGGC GTTCGcTagCAGTGGAGAC-3') as a 1,308-bp fragment. The PCR product was digested with NheI and SphI (sites underlined) and ligated into the same sites in pBAD18-Km to make plasmid pWQ72.

PAGE analysis. For polyacrylamide gel electrophoresis (PAGE) analysis, LPS was isolated from proteinase K-digested whole-cell lysates, as described by Hitchcock and Brown (19). The LPS was then separated on 4 to 12% Bis-Tris NuPAGE gels or 10 to 20% Tricine gels from Invitrogen and visualized by silver staining (65). For sodium dodecyl sulfate (SDS)-PAGE of proteins, the protein samples were solubilized in SDS-containing sample buffer (31) by boiling at 100°C for 15 min and then were separated on 12% SDS-PAGE gels. Proteins were visualized by Coomassie brilliant blue staining or by Western immunoblotting. For Western immunoblotting, proteins were transferred to nitrocellulose membranes (Pall Life Sciences) and probed with QIAGEN anti-pentahistidine mouse monoclonal antibodies according to the manufacturer's instructions. Colorimetric detection was used with a secondary goat anti-mouse antibody conjugated to alkaline phosphatase (Jackson ImmunoResearch Laboratories Inc.), visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate.

Gl_{KP} purification. The Gl_{KP} protein was overexpressed from pWQ67 in *E. coli* BL21[λ DE3]. This strain was grown at 37°C for 18 h in LB supplemented with kanamycin. The culture was then diluted 1:100 in 100 ml of fresh medium, and incubation was continued until the culture reached an optical density of 0.6 at 600 nm. Expression of His₆-Gl_{KP} was induced by adding isopropyl-1-thio- β -D-galactopyranoside to the culture at a final concentration of 1 mM and continuing incubation for another 3.5 h. The cells were harvested, washed once in buffer A (50 mM NaH₂PO₄, pH 8.0, containing 300 mM NaCl) containing 10 mM imidazole, and then the pellet was frozen until required. The cell pellet was resuspended in 7 ml of buffer A containing 10 mM imidazole, and the cells were lysed by sonication. Unbroken cells and cell debris were removed by centrifugation (10 min at 20,000 $\times$ g). The membrane fraction was removed from the cleared lysate by ultracentrifugation (60 min at 100,000 $\times$ g). The supernatant was combined with 1 ml of 50% Ni²⁺-nitrilotriacetic acid (NTA) suspension (QIAGEN), and the mixture was incubated for 2 h at 4°C on a rotary shaker. The lysate-Ni²⁺-NTA mixture was then loaded into a disposable plastic column (5 ml) with elution by gravity flow. The column was washed twice with 10 column volumes of buffer A containing 15 mM imidazole. The protein was eluted with buffer A containing 250 mM imidazole, and approximately 2 ml of eluate was collected in 0.5-ml fractions. The fractions were examined by SDS-PAGE, and those with the highest amount of highest purity protein were pooled and dialyzed against 20 mM Tris-HCl, pH 8.0. AEBSEF [4-[2-aminoethyl] benzenesulfonyl fluoride hydrochloride] (Sigma) protease inhibitors were added to a final con-

centration of 1 mM. The purified enzyme was stored at -80°C in 20% glycerol, without any loss of activity. Typical extracts contained 3 to 4 mg/ml protein.

Enzymatic activity of Gl_{KP}. The enzymatic activity of Gl_{KP} was assayed using a protocol adapted from Creuzenet et al. (8). Unless otherwise stated, the reaction mixture comprised 20 mM Tris-HCl, pH 8.0, buffer, containing 1 mM UDP-GlcUA substrate and 96.0 ng of purified Gl_{KP} in a total of 0.05 ml. Reactions were carried out at 37°C and stopped by transfer of the reaction tubes to a boiling water bath for 6 min. The reaction products were separated by capillary electrophoresis (CE) using a Beckman P/ACE 5000 system (Beckman Instruments, Fullerton, CA) with UV detection at 254 nm. The running buffer was 25 mM sodium tetraborate, pH 9.5, and a bare silica capillary (75 μ m by 57 cm) was used with a detector at 50 cm. The capillary was washed before each run for 2 min with 0.2 M NaOH, 2 min with water, and 4 min with running buffer. Samples were injected by pressure for 8 s and then separated at 22 kV. The Beckman P/ACE software was used for peak integration. For kinetic analyses, reactions were performed in a total of 0.05 ml of 20 mM Tris-HCl containing a range of 0.01 to 1.00 mM UDP-GlcUA and 12.0 ng of purified enzyme at 37°C for 3 min and then heated at 95°C for 6 min to quench the reaction. The initial rate conditions were selected to give less than 10% substrate conversion in 3 min. The reaction mixtures were analyzed by CE, and the kinetic parameters K_m and the maximum rate of metabolism (V_{max}) were calculated using the PRISM software (GraphPad Software Inc., San Diego, CA).

Properties of Gl_{KP}. The molecular weight of purified His₆-Gl_{KP} was determined by matrix-assisted laser desorption/ionization-time of flight mass spectrometry performed at the Biological Mass Spectrometry Facility, University of Guelph. The oligomerization status of Gl_{KP} was determined on a Superose 12 HR 10/30 column (Amersham Biosciences) equilibrated with 50 mM Tris, pH 8.0, containing 100 mM NaCl. Protein elution was monitored at a wavelength of 280 nm. The column was calibrated with Sigma molecular mass standards (12.4 to 200 kDa). Two injections were carried out with a total of 50 μ g and then 200 μ g of His-Gl_{KP} in a final volume of 0.2 ml.

In order to release NAD⁺/NADH bound by the enzyme, a total of 240 μ g of Gl_{KP} in 0.05 ml was treated with 10 μ g of proteinase K for 1 h at 37°C. The products were analyzed by CE and compared to commercially available NAD⁺ and NADH standards available from Sigma. To verify the presence of NAD⁺ and NADH, one sample of proteinase K-digested Gl_{KP} was spiked with 0.2 mM NAD⁺ and NADH.

The pH dependence was measured under standard assay conditions using 20 mM sodium acetate (pH 4.5, 5.0, and 5.5), 20 mM morpholineethanesulfonic acid (pH 6.0 and 6.5), 20 mM Tris-HCl (pH 7.0, 7.5, 8.0, 8.5, and 9.0), and 20 mM glycine-KOH (pH 9.5, 10.0, and 10.5) over 2 h. The temperature optimum was determined under standard assay conditions by measuring the amount of product

FIG. 2. Overexpression and purification of His₆-Gla_{KP}. (A) Coomassie blue-stained SDS-PAGE of the cell-free lysate, soluble fraction, and membrane fractions of *E. coli* BL21[λDE3] [pET28(+)] and *E. coli* BL21[λDE3] (pWQ67). Loading was adjusted to correspond to the original cell-free lysate so that the relative amounts of His₆-Gla_{KP} (4.3 μg) can be compared directly. (B) SDS-PAGE analysis of purified His₆-Gla_{KP} after Ni²⁺-NTA affinity chromatography. His₆-Gla_{KP} has a predicted size of 39 506.81 Da.

formed in 2 h at 12, 20, 25, 30, 37, 45, 50, 55, and 65°C. To study the requirement for NAD⁺, reactions were carried out under standard conditions for 2 h with or without NAD⁺ (at a 1 mM final concentration). The influence of divalent cations (MgCl₂, MnCl₂, and CaCl₂) was tested at final concentrations of 0 to 20 mM final concentrations.

HPAEC analysis of the Gla_{KP} reaction products. The standard Gla_{KP} assay was scaled up to a 0.25-ml reaction mixture containing 4 mM UDP-GlcUA substrate and 24.0 μg of purified Gla_{KP} in 20 mM Tris-HCl, pH 8.0. Reactions were carried out for 2 h at 37°C and then stopped by hydrolysis of the UDP moiety. The samples were acidified to pH 2 with 0.05 ml of 0.1 N HCl, boiled for 5 min, and then neutralized with 0.05 ml of 0.1 N NaOH. The reaction products were then treated with 2 μl of alkaline phosphatase (20,000 U/ml; Amersham Pharmacia) for 2 h at 37°C. Protein was removed from the sample by filtration through a Microcon YM-3 0.5-ml centrifugal filter device (NMWL 3,000 Da; Millipore). Monosaccharides were then separated on a CarboPac PA1 column (4 mm by 250 mm; Dionex) by high-performance anion-exchange chromatography (HPAEC) on a Dionex BioLC system equipped with an electrochemical detector. Samples were resuspended in 10 mM NaOH, and a total of 10 nmol was injected. The column was washed for 2 min with 150 mM NaOH after injection, and the monosaccharides were eluted with a linear gradient of 0 to 25% 1 M sodium acetate in 150 mM NaOH over 38 min.

RESULTS

Identification of Gla_{KP}. A putative UDP-GalUA C4-epimerase (Gla_{EC}; WbnF) was identified in the O-PS biosynthesis gene cluster of *E. coli* O113 (45). The sequence of this open reading frame was used for a BLAST search of the unfinished genome of the clinical isolate *K. pneumoniae* MGH 78578 (<http://genome.wustl.edu/projects/bacterial/kpneumoniae/>). An open reading frame on CONTIG705 was identified showing 82% identity and 93% similarity at the amino acid level to Gla_{EC} (WbnF) (accession number AAD50494.1). A portion of the *K. pneumoniae* homolog of Ugd was also encoded on CONTIG705. A genomic region of *K. pneumoniae* NTUH-K2044 was recently sequenced that contained genes involved in exopolysaccharide synthesis, sugar nucleotide precursor synthesis, and O-PS biosynthesis and export (11). The *gla_{KP}* gene was

found on this segment, although not annotated by the authors, and is located downstream of *ugd* and transcribed in the opposite orientation. Downstream of *gla_{KP}* is a hypothetical protein of unknown identity, followed by the O-PS biosynthetic cluster.

As this work was in progress, the *K. pneumoniae gla_{KP}* gene (named *uge* by the authors) of *K. pneumoniae* O1:K2 was identified by another group of investigators in a screen for mutants affecting capsule expression (52). They showed that a *gla_{KP}* mutant strain lacked UDP-GalUA, but the Gla_{KP} enzyme was not characterized.

Gla_{KP} shares identity with several nucleotide sugar epimerases encoded by organisms containing GalUA-containing polymers. For example, it shares 53% identity and 67% similarity to Gla_{SM} (LpsL) (accession number CAA10917.1) of *Sinorhizobium meliloti* (26, 27), 50% identity and 67% similarity to Gla_{SP} (Cap1J) (accession number CAB05928) of *S. pneumoniae* (37, 38), and 47% identity and 64% similarity to GAE1 (accession number AAT77233) of *Arabidopsis* (35). The functions of all of these enzymes as UDP-GalUA epimerases have been shown in vitro (26, 35, 37).

Gla_{KP} expression and purification. The Gla_{KP} protein has a predicted molecular mass of 37,343.57 Da and a slightly acidic pI of 5.39. The His₆-Gla_{KP} protein has a predicted molecular mass of 39,506.81 Da, consistent with that determined by matrix-assisted laser desorption ionization–time of flight mass spectrometry analysis of the purified protein (39,524.88 Da). By SDS-PAGE analysis, most of the protein is present in the soluble fraction (Fig. 2A). It was expressed at approximately 3 to 4 mg/100 ml of culture, representing 49.9% of the total protein (Table 2). Gla_{KP} was purified to near homogeneity by nickel chelation chromatography (Fig. 2B). The presence of the N-terminal His₆ tag was verified by Western immunoblot-

TABLE 2. Purification table for Gla_{KP} as determined by CE

Fraction	Volume (ml)	Protein concn (mg/ml)	Total protein (mg)	Yield (%)	Total activity ^a (u)	Specific activity (u/mg)	Recovery (%)	Purification factor
Total cell extract	6.5	4.60	29.9	100	4.87	0.163	100	1
Soluble fraction	6.4	3.57	22.8	76.3	4.43	0.194	91.0	1.19
Ni ²⁺ -NTA affinity chromatography (after dialysis)	2.39	4.54	10.9	36.5	2.43	0.223	49.9	1.37

^a One unit is defined the amount of enzyme required to convert 1 μ mole of substrate in 1 min under our experimental conditions. Assay reactions were performed in a total of 50 μ l with 1 mM UDP-GlcUA and 2 μ l of a 1/10 dilution of each enzyme fraction for 1 min at 37 °C. The reactions were stopped by boiling for 5 min and the activity was determined by CE.

ting with an anti-pentahistidine monoclonal antibody (data not shown).

Gel filtration chromatography showed that Gla_{KP} has an apparent molecular mass of approximately 75,000 Da (data not shown), consistent with a dimeric structure.

HPAEC analysis of the Gla_{KP} reaction products. To confirm the predicted activity of Gla_{KP}, the reaction products were first treated with HCl and then dephosphorylated prior to HPAEC analysis. The resulting monosaccharides were readily separated by HPAEC (Fig. 3, trace A). Two peaks were identified

in the reaction mixtures with elutions consistent with GlcUA and GalUA standards (Fig. 3, traces B and C, respectively). This analysis was consistent with the predicted UDP-GalUA C4-epimerase activity of Gla_{KP}, converting UDP-GlcUA to UDP-GalUA. This reaction is likely reversible, as is the case with Gla_{SP} (Cap1J) (37), but this was not tested since UDP-GalUA is no longer commercially available.

CE analysis of Gla_{KP} activity. The products of Gla_{KP} activity were analyzed by CE, which showed the time-dependent appearance of a novel peak migrating faster than the UDP-GlcUA substrate peak. The product was determined to be UDP-GalUA by HPAEC analysis (Fig. 4A). At equilibrium, under these experimental conditions, Gla_{KP} activity appeared to favor UDP-GalUA formation with a ratio of about 1.4:1 for the conversion of UDP-GlcUA to UDP-GalUA (Table 3). Time course experiments were carried out with different enzyme dilutions, showing a dependence of Gla_{KP} activity on both time and enzyme concentration (Fig. 4B).

Biochemical characterization of Gla_{KP}. Gla_{KP} has activity over a wide pH range with an optimum pH of ~7.5 to 8.0 (data not shown). It is also active over a broad range of temperatures, with an optimum between 30 to 45°C (data not shown). The addition of exogenous NAD⁺ had no effect on enzyme activity (Table 3), as was the case with Gla_{SP} (Cap1J) from *S. pneumoniae* (37). However, NAD⁺ is likely an essential coenzyme, as it was shown to be required for the mechanism of C4 epimerization described for GalE. In GalE isolated from *E. coli* or yeast cells, NAD⁺ is tightly bound to the enzyme and does not dissociate (reviewed in reference 15). Therefore, Gla_{KP} was digested with proteinase K to release any bound cofactor. Peaks corresponding to NAD⁺ and NADH were seen on the CE spectrum of the digestion products (data not shown), indicating that NAD⁺ is also bound by Gla_{KP}. Divalent cations had an unexpected effect on Gla_{KP} activity (Fig. 4C). CaCl₂, MgCl₂, and MnCl₂ were all inhibitory toward conversion of UDP-GlcUA to UDP-GalUA. The most drastic effect was seen with MnCl₂, which caused total inhibition at a concentration of 5 mM. This type of effect has not been seen with other epimerases. For example, the UDP-Gal C4-epimerase GalE from *E. coli* (70) and the UDP-GlcNAc C4-epimerase WbpP of *Pseudomonas aeruginosa* (8) showed no change in activity with the addition of divalent cations.

The *K_m* and *V_{max}* values of Gla_{KP} were determined from a Michaelis-Menten plot. Gla_{KP} has a substantially higher affinity for the substrate UDP-GlcUA and is a more efficient enzyme than Gla_{SP} (Cap1J) (Table 4). However, it is difficult to make detailed comparisons between the kinetic properties of

FIG. 3. HPAEC chromatogram of Gla_{KP} reaction products after hydrolysis to release the UDP-moiety. Assay mixtures containing 4 mM UDP-GlcUA substrate and 24.0 μ g of purified Gla_{KP} were incubated for 2 h. The mixtures were treated with HCl to release the monosaccharides and then incubated with alkaline phosphatase. After removal of protein, the reaction products (A) were separated on a CarboPac PA1 column with a linear gradient of 0 to 25% 1 M sodium acetate. GlcUA (B) and GalUA (C) standards were run to confirm the identity of the reaction products.

FIG. 4. Epimerization of UDP-GlcUA by Gla_{KP} at equilibrium. The reactions were carried out in a total volume of 50 μ l with 1 mM UDP-GlcUA and incubated at 37°C. Panel A shows the CE spectrum of assay mixtures after variation in incubation times. The upper trace is a control reaction containing no enzyme. Panel B demonstrates the dependence of Gla_{KP} activity and product formation on time and enzyme concentration. Panel C illustrates the effect of divalent cations on Gla_{KP} activity. Reactions were incubated for 2 h at 37°C with 1 mM UDP-GlcUA and 96.0 ng of Gla_{KP}. AU, arbitrary units.

the two enzymes, since they differ in enzyme stability, in the homogeneity of the protein preparations, and in the assays used to determine the kinetic parameters.

Relaxed substrate specificity of Gla_{KP}. In addition to UDP-GlcUA, Gla_{KP} was also found to be capable of reversible interconversion of UDP-Glc and UDP-Gal, as well as the acetamido derivatives UDP-GlcNAc and UDP-GalNAc (Table 3). The rate of conversion of UDP-Gal was much higher than

that for UDP-Glc, with 79.8% UDP-Gal converted to UDP-Glc and only 38.9% UDP-Glc transformed to UDP-Gal at equilibrium with equivalent amounts of enzyme (data not shown). A shift toward UDP-Glc production at equilibrium was also reported for GalE (70). In contrast, the maximal conversion rates for UDP-GlcNAc and UDP-GalNAc were much lower, at 9.5% and 32.1% for UDP-GlcNAc and UDP-GalNAc, respectively. Interestingly, the amount of conversion of UDP-Gal to UDP-Glc was also higher than that of the optimal substrate UDP-GlcUA at the same enzyme concentration. A reaction time course showed that the conversion of UDP-Glc and UDP-Gal was time dependent (data not shown). This broader specificity distinguished Gla_{KP} from Gla_{SP} (Cap1J) since the latter showed no activity with either UDP-Glc or UDP-Gal (37). The kinetic parameters for Gla_{KP} using UDP-Glc and UDP-Gal as substrates could not be determined because the enzyme was still not saturated at substrate concentrations of 24 mM (data not shown). This suggests that neither is likely to be an ideal substrate for the enzyme.

Examination of Gla_{KP} activity in vivo. In order to examine the biological relevance of the action of Gla_{KP} on UDP-Glc and UDP-Gal, *gla_{KP}* was introduced on a plasmid into a *S. enterica* serovar Typhimurium *galE* mutant strain (Fig. 5).

TABLE 3. Substrate specificity and NAD⁺ requirement of Gla_{KP}^a

Substrate	Product formation	
	% UDP-GalUA	% UDP-GlcUA
UDP-GlcUA	57.3 $\pm$ 0.4	42.7 $\pm$ 0.4
UDP-GlcUA plus NAD ⁺	57.3 $\pm$ 0.3	42.7 $\pm$ 0.3
	% UDP-Glc	% UDP-Gal
UDP-Glc	79.8 $\pm$ 0.15	20.2 $\pm$ 0.15
UDP-Gal	61.1 $\pm$ 0.2	38.9 $\pm$ 0.2
	% UDP-GlcNAc	% UDP-GalNAc
UDP-GlcNAc	90.5 $\pm$ 0.5	9.5 $\pm$ 0.5
UDP-GalNAc	32.0 $\pm$ 0.7	68.0 $\pm$ 0.7

^a Each experiment was performed in triplicate.

TABLE 4. Kinetic parameters of UDP-GalUA C4-epimerases from *K. pneumoniae* and *S. pneumoniae*

Enzyme	Substrate	K_m (μ M)	V_{max} (pmol/min)	Enzyme (pmol)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{mM}^{-1} \times \text{min}^{-1}$)
Gla _{KP} ^a	UDP-GlcUA	13.0 $\pm$ 0.8	113.0 $\pm$ 1.45	0.304	372 $\pm$ 4.78	2.86 $\times 10^4 \pm 0.72 \times 10^4$
Gla _{SP} ^b (UDPGLE; Cap1J)	UDP-GlcUA	240	7.5	124	0.0605	0.25

^a The results for Gla_{KP} are derived from four separate experiments done in triplicate.
^b The K_m for *S. pneumoniae* Gla_{SP} (Cap1J) (37) was reported in the literature. The V_{max} was determined from the Lineweaver-Burk plots presented in the study, and the values for k_{cat} and k_{cat}/K_m were calculated in order to compare them to the values obtained for Gla_{KP}.

GalE is a well-characterized reversible UDP-Gal C4-epimerase involved in Gal metabolism. The core OS of *S. enterica* serovar Typhimurium contains Gal (21). When grown in minimal medium supplemented with Glc, *S. enterica* serovar Typhimurium SL1306 (*galE*) produces a truncated core OS that can no longer ligate O-PS; however, when Gal is present in the medium, UDP-Gal can be synthesized directly (rather than from epimerization of UDP-Glc), and core OS elongation and O-PS ligation are restored. When *gla*_{KP} was expressed in SL1306 (pWQ69; Gla_{KP}⁺), partial complementation of the mutation was evident (Fig. 5A, lane 5), consistent with the synthesis of a wild-type *S. enterica* serovar Typhimurium core OS, although this was not confirmed by structural studies. The presence of Glc in the medium would prevent full induction of

*gla*_{KP} by arabinose, but even under these suboptimal conditions the data clearly show that Gla_{KP} can carry out the synthesis of UDP-Gal at physiological concentrations of UDP-Glc. The results in *S. enterica* serovar Typhimurium raised the question of whether Gla_{KP} performed dual functions in *K. pneumoniae*. To address this, a *galE* mutant (CWG631) was constructed in *K. pneumoniae*. The *galE* gene was identified by a BLAST search of the unfinished genome of *K. pneumoniae* MGH 78578 (<http://genome.wustl.edu/projects/bacterial/kpneumoniae/>) based on its homology with the *galE* gene of *E. coli* K-12 (NP_451280.2). Primers were designed using the *K. pneumoniae* MGH 78578 sequence to PCR amplify the *galE* gene from *K. pneumoniae* CWK2. The nucleotide sequence of the 5' region of

FIG. 5. NuPAGE gels (10 to 12%) showing the complementation of *galE* mutations in *S. enterica* serovar Typhimurium (SL1306) and in *K. pneumoniae* (CWG631) with pWQ69 (Gla_{KP}⁺). Gene expression from complementing plasmids was induced by the addition of 0.6% arabinose to the medium. (A) PAGE analysis of *S. enterica* serovar Typhimurium mutant SL1306 and complementation with pWQ69 (Gla_{KP}⁺). These strains were grown in M9 minimal medium supplemented with 0.4% glucose. In order to restore synthesis of the full-length core OS and O-PS addition in SL1306, 0.4% Gal was added. (B) PAGE analysis of *K. pneumoniae* CWG631 (*galE*) and complementation with pWQ72 (GalE⁺) and pWQ69 (Gla_{KP}⁺). These strains were grown in LB and, in some cases, were supplemented with 0.4% Glc to repress Gal uptake from the medium.

the *galE* gene from *K. pneumoniae* CWK2 is 98% identical to the corresponding region of the *galE* gene from strain CG43 (M94964.1; the 3' end of the CG43 *galE* has not been sequenced) (46). The activity of the *galE* gene product from *K. pneumoniae* CG43 has been characterized by complementation analysis (46). The GalE gene product of *K. pneumoniae* CWK2 shows 94% identity and 97% similarity and 92% identity and 97% similarity to GalE from *S. enterica* serovar Typhimurium (NP_459755.1) and *E. coli* K-12 (NP_451280.2), respectively. The O-PS of *K. pneumoniae* O1 CWK2 is a polymer of disaccharide repeat units containing galactopyranose and galactofuranose (29, 30, 68). The *galE* mutant (CWG631) produces full-length core OS lacking O-PS (Fig. 5B), consistent with the absence of UDP-Gal C4-epimerase. When Gal was added to the culture medium, O-PS synthesis was not restored. This can be explained by the anticipated polar effects of the *galE::PRE112* insertion mutation on *galK*. The *gal* operon is synthesized from a polycistronic mRNA in the order of *galE* (epimerase), *galT* (galactose-1-phosphate uridylyltransferase), and *galK* (galactokinase), as in *E. coli* and *Salmonella* (46). As expected, in the presence of GalE from a complementing plasmid, UDP-Gal formation can still occur and O-PS synthesis is restored, independent of GalK activity (Fig. 5B, lanes 4 and 5). CWG631 (*galE*) produces rough LPS, indicating that the chromosomal copy of *gla_{KP}* cannot compensate for the mutation in *galE*. When pWQ69 (*Gla_{KP}*⁺) was transformed into CWG631 (*galE*), only a slight amount of O-PS was added to the core OS (Fig. 5B, lane 9). Despite the small amount of O-PS formed under conditions where *Gla_{KP}* is overexpressed in *K. pneumoniae* CWG631 (data not shown), the rough LPS phenotype of CWG631 (*galE*) provides convincing evidence that under physiological conditions, *Gla_{KP}* activity cannot substitute for GalE to any significant extent.

The differences in the extent of *Gla_{KP}* complementation of *galE* mutants in *S. enterica* serovar Typhimurium and *K. pneumoniae* may be explained by the amount of Gal required in each strain. Only two Gal residues are required for core OS completion in *S. enterica* serovar Typhimurium, whereas a large amount of Gal is required for synthesis of the polygalactan O-PS repeat unit in *K. pneumoniae*.

DISCUSSION

Gla_{KP} plays an important role in the virulence of *K. pneumoniae*. Mutants in *gla_{KP}* (*uge*) show enhanced susceptibility to hydrophobic compounds (52; E. Fridrich, E. Vinogradov, and C. Whitfield, unpublished results). However, correlating this phenotype to avirulence is not possible because the position of GalUA in the core OS backbone (Fig. 1) means that a *gla_{KP}* mutant cannot ligate O-PS. In addition, the *gla_{KP}* mutant strain did not produce capsular polysaccharide, even though the capsule structure of the wild-type K2 strain used to construct the mutant does not contain GalUA. Both O-PS and capsular polysaccharide are critical virulence determinants in *K. pneumoniae* (49), and the avirulence of the *gla_{KP}* mutant could be due to either (or both) of these defects.

Proteins with UDP-GalUA C4-epimerase activities have been described in *S. pneumoniae* (Cap1J; *Gla_{SP}*) (37), *S. meliloti*, (LpsL; *Gla_{SM}*) (26), and in *Arabidopsis* (GAE1) (35). All of these enzymes have been shown to be UDP-GalUA epimerases in *in vitro* enzyme assays. *Gla_{SP}* (Cap1J) was partially

purified and was the first UDP-GalUA epimerase to be characterized at a biochemical level (37). However, attempts to purify *Gla_{SP}* to electrophoretic homogeneity without loss of activity were unsuccessful. Biochemical characteristics are also available for GAE1 from *Arabidopsis*, although purified protein was not used in these assays (35). Interestingly, unlike bacterial UDP-GalUA C4-epimerases, the plant enzymes are predicted to be membrane proteins likely targeted to the Golgi, where they would supply UDP-GalUA to Golgi transferases involved in pectin biosynthesis (35).

Gla_{KP} shares a high level of sequence similarity with nucleotide sugar epimerases and other members of the short-chain dehydrogenase/reductase (SDR) enzyme family of oxidoreductases (reviewed in references 25 and 44). The SDR family currently includes over 3,000 enzymes from all forms of life. Typically, they carry out oxidation-reduction reactions, usually functioning as dehydrogenases, dehydratases, isomerases, or epimerases. These enzymes share anywhere from 15 to 30% amino acid identity and display two conserved motifs (25, 44) that are present in *Gla_{KP}* (Fig. 6). The first is a TGXXGXXG motif found in the N terminus which displays a conserved alternating α/β -folding pattern typical of a Rossmann fold involved in coenzyme (NAD⁺) binding (13, 53, 69). The second motif consists of the catalytic triad of Ser, Tyr, and Lys with the YXXK motif, which has recently been extended to a catalytic tetrad of Asn, Ser, Tyr, and Lys (13). The Ser residue may be replaced by Thr in some members of the SDR family.

Gla_{KP} shows 24% identity and 43% similarity at the amino acid level to *E. coli* GalE (accession number NP_415280). Secondary structure predictions of *Gla_{KP}* (data not shown) using PSIPred analysis (<http://bioinf.cs.ucl.ac.uk/psipred/>) (24) predict *Gla_{KP}* to have a secondary structure very similar to that of the GalE enzyme of *E. coli* (3). GalE is subdivided into two domains with the N-terminal domain containing the typical Rossmann fold with six β -sheets surrounded by α -helices and a seventh β -sheet in the C-terminal domain. The small C-terminal domain of GalE is involved in substrate binding, and this is probably also the case with *Gla_{KP}*, based on overall structural similarity. The cleft between the two domains contains the active site (59–61). *Gla_{KP}* was found to be a dimer by gel filtration, as is the case with *Gla_{SP}* (Cap1J) (37), the UDP-GlcNAc epimerase WbpP of *P. aeruginosa* (8), and GalE from both *E. coli* (3) and *Homo sapiens* (63). In the GalE prototype, each subunit of the homodimer contains one molecule of NAD⁺ and one molecule of UDP-linked substrate (3, 63). NAD⁺ addition to *Gla_{KP}* enzyme reactions had no effect on enzyme activity, but this cofactor was found to be bound to the enzyme, as was the case with *Gla_{SP}* (Cap1J) from *S. pneumoniae* (37). The mechanism by which GalE catalyzes the C4-epimerization of UDP-Glc/UDP-Gal has been well characterized (1, 12, 15, 20). It involves formation of a transient keto-sugar intermediate with transient reduction of enzyme-bound NAD⁺. The mechanism by which *Gla_{KP}* mediates the C4-epimerization of UDP-GlcUA/UDP-GalUA may be similar, given the overall amino acid similarity showed by GalE (from *E. coli*) and *Gla_{KP}*, particularly the conservation of active site residues.

Gla_{KP} UDP-GalUA C4-epimerase activity favors the formation of UDP-GalUA formation in a ratio of 1.4:1, which is similar to the equilibrium constant of 1.3 in the direction of

FIG. 6. Alignment of *Gla*_{KP}, its bacterial homologs, and other characterized bacterial epimerases. The *K. pneumoniae* *Gla*_{KP} was aligned with its homologs in *E. coli* O113 WbnF, *S. meliloti* LpsL, and *S. pneumoniae* Cap1J, as well as the UDP-GlcNAc epimerase WbpP from *P. aeruginosa* and the UDP-Gal epimerases from *E. coli* K-12 (designated K-12Gale) and from *Homo sapiens* (designated HSGale). Crystal structures are available for these three enzymes (3, 23, 64). Identical amino acids are shown in black, and similar residues are boxed in gray. Underlined residues are involved in nucleotide binding (53, 69), and amino acids marked by an asterisk have been shown to be important for catalysis (13, 25, 44). Amino acids that are underlined twice are thought to be involved in substrate binding (23). Multiple alignments were performed with CLUSTAL_W available at the Expasy molecular biology server (au.expasy.org).

UDP-GalUA formation reported for *S. pneumoniae* Gl_{SP} (Cap1J) (37) and *Arabidopsis* GAE1 (35). However, Gl_{SP} and GAE1 could not interconvert UDP-Glc/UDP-Gal (35, 37).

Some epimerases are known to have expanded substrate specificity, although this property has not been previously reported for a UDP-GalUA 4-epimerase. Interestingly, the mammalian form of GalE, unlike that of *E. coli*, can turn over UDP-GlcNAc and UDP-GalNAc in addition to UDP-Glc/UDP-Gal. This has been attributed to a larger (by approximately 15%) saccharide-binding site in the human enzyme and an amino acid substitution in the active site from Tyr299 in *E. coli* to Cys307 in the human form (Fig. 6, the last doubly underlined residue). This facilitates the accommodation of the *N*-acetyl group at the C2 position of the sugar (62, 64). It has also been shown that the Gne enzyme of *Bacillus subtilis* interconverts UDP-Glc and UDP-Gal, as well as (to a lesser extent) the *N*-acetylated forms of the substrates (58). These activities are seen in vitro and in vivo. The Gne enzyme of *Campylobacter jejuni* was recently shown to be a bifunctional UDP-Glc/GlcNAc epimerase with both in vitro and in vivo activity (5).

Several studies now suggest that the Tyr299 in *E. coli* GalE (Cys307 in the human GalE) is important in determining the size of the active site cleft, with smaller residues enabling the interconversion of larger substrates with or without compromising the conversion of smaller substrates (4, 23, 57, 62, 64). This residue has been termed a form of "gatekeeper" (57). The UDP-GlcNAc 4-epimerases WbpP from *P. aeruginosa* serotype O6 (8) and Gne from *Yersinia enterocolitica* serotype O8 (4) also have weak UDP-Gal 4-epimerase activity. A recent determination of the crystal structure of WbpP with UDP-GlcNAc and UDP-Glc reexamined the basis of substrate specificity of UDP-hexose C4-epimerases (23). In the case of WbpP, orbital steering is proposed to explain the preferential catalysis of UDP-GlcNAc/UDP-GalNAc over UDP-Glc/UDP-Gal (23, 32). The saccharide-binding pocket of WbpP is larger than that of GalE, due to the substitution of certain amino acids in the binding pocket (Fig. 6, double-underlined residues). According to the model, this allows water molecules to play a role in saccharide binding. The *N*-acetyl group permits the ordering of water molecules in the active site, thus stabilizing the saccharide group for catalysis. The same is not seen in nonacetylated substrates, and their conversion is not as efficient. Alignments of Gl_{KP} homologs with WbpP and GalE show that the residues thought to be involved in the saccharide-binding pocket are conserved between UDP-GalUA 4-epimerases (including *Arabidopsis* GAE1 [data not shown]). However, there are differences compared to the corresponding residues present in WbpP and GalE (Fig. 6, underlined residues). The presence of Ala86, Thr180, and Thr298 in UDP-GalUA epimerases would potentially influence the size of the saccharide-binding pocket. It is therefore plausible that the preferential catalysis of UDP-GlcUA over UDP-Glc/UDP-Gal and UDP-GlcNAc/UDP-GalNAc epimerization may also reflect orbital steering. Nuclear magnetic resonance studies have shown that divalent cations (Mg²⁺, Mn²⁺, and Ca²⁺) may be involved in preorganizing the nucleotide sugar in enzymatic reactions (36). Binding studies on *E. coli* GalE have shown strong binding of the UDP group to the protein (reviewed in reference 15). The inhibitory action of divalent cations on Gl_{KP} activity may be

due to their influence on positioning of the nucleotide sugar in the active site.

While epimerases have been shown to act on various substrates in vitro, this activity is not always biologically relevant. For example, the UDP-Gal 4-epimerase activity of WbpP has not been detected under physiological conditions in *P. aeruginosa* (8). The *Y. enterocolitica* Gne enzyme was able to complement an *E. coli* K-12 *galE* mutation, but the importance of this activity in *Y. enterocolitica* was not established. *Y. enterocolitica* O8 does possess a "true" *galE* gene elsewhere on its chromosome (47). In the case of Gl_{KP} of *K. pneumoniae*, UDP-Gal 4-epimerization activity could be demonstrated in vitro but does not play a significant role in vivo in *K. pneumoniae*.

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