Characterization of *Vibrio cholerae* O1 El Tor *galU* and *galE* Mutants: Influence on Lipopolysaccharide Structure, Colonization, and Biofilm Formation

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Recently we described the isolation of spontaneous bacteriophage K139-resistant *Vibrio cholerae* O1 El Tor mutants. In this study, we identified phage-resistant isolates with intact O antigen but altered core oligosaccharide which were also affected in galactose catabolism; this strains have mutations in the *galU* gene. We inactivated another *gal* gene, *galE*, and the mutant was also found to be defective in the catabolism of exogenous galactose but synthesized an apparently normal lipopolysaccharide (LPS). Both *gal* mutants as well as a rough LPS (R-LPS) mutant were investigated for the ability to colonize the mouse small intestine. The *galU* and R-LPS mutants, but not the *galE* mutant, were defective in colonization, a phenotype also associated with O-antigen-negative mutants. By investigating several parameters in vitro, we could show that *galU* and R-LPS mutants were more sensitive to short-chain organic acids, cationic antimicrobial peptides, the complement system, and bile salts as well as other hydrophobic agents, indicating that their outer membrane no longer provides an effective barrier function. O-antigen-negative strains were found to be sensitive to complement and cationic peptides, but they displayed significant resistance to bile salts and short-chain organic acids. Furthermore, we found that *galU* and *galE* are essential for the formation of a biofilm in a spontaneous phage-resistant rugose variant, suggesting that the synthesis of UDP-galactose via UDP-glucose is necessary for biosynthesis of the exopolysaccharide. In addition, we provide evidence that the production of exopolysaccharide limits the access of phage K139 to its receptor, the O antigen. In conclusion, our results indicate involvement of *galU* in *V. cholerae* virulence, correlated with the observed change in LPS structure, and a role for *galU* and *galE* in environmental survival of *V. cholerae*.

The causative agent of the intestinal disease cholera is *Vibrio cholerae*, a gram-negative motile bacterium. Of the more than 150 known serogroups, only the noncapsulated O1 and the encapsulated O139 serogroup have been found to be associated with epidemic cholera. Epidemic O139 strains are related to and were derived from O1 El Tor strains after genetic alterations of the O-antigen biosynthesis gene cluster (16). The ongoing seventh pandemic, which began in 1961, is caused by O1 El Tor strains (3). *V. cholerae* is a natural inhabitant of aquatic ecosystems and is known to attach to environmental surfaces such as plants, filamentous green algae, zooplankton, crustaceans, or insects (8). Recently, *V. cholerae* O1 El Tor was found to form a three-dimensional biofilm on abiotic surfaces (70). Biofilm formation may be important in the life cycle of pathogenic *V. cholerae* strains, because they reside within natural aquatic habitats during interepidemic periods. O1 El Tor strains are also able to switch to a rugose colony phenotype. This morphology correlates with the constitutively production of an exopolysaccharide allowing biofilm formation on abiotic surfaces (65, 72). Such rugose variants are chloride resistant and fully virulent in humans (43).

Important steps of the noninvasive disease process include ingestion of *V. cholerae* along with contaminated food or water, passage through the gastric acid barrier of the stomach, adherence to and penetration through the intestinal mucus lining, adherence to intestinal epithelial cells, multiplication, and cholera toxin (CT) production (25). In the course of the transition from the external environment to the human body, the bacteria are exposed to a series of changes, such as in temperature, acidity, and osmolarity, and must survive in the intestine, an environment which is enriched in growth-inhibitory substances like bile salts and organic acids. They are also exposed to antimicrobial components of the innate immune system, like complement factors secreted by intestinal epithelial cells (2) or defensins produced by Paneth cells (37). Several *V. cholerae* gene products have been shown to be important for colonization of the small intestine. These include the toxin-coregulated pilus (TCP) (62), accessory colonization factors (49), regulatory proteins (e.g., ToxR/ToxS, TcpP/TcpH, and ToxT) and outer membrane proteins (21, 55), metabolic factors, biotin, purine biosynthetic genes, and the O antigen of the lipopolysaccharide (LPS) (6). Some factors are suggested to be involved in adhesion to the small intestine; however, no specific *V. cholerae* adhesins or specific mucosal receptors have been identified.

In gram-negative bacteria, LPS is the major integral component of the outer membrane with three general features: O antigen, core oligosaccharide, and lipid A. The O antigen of *V. cholerae* O1 consists of a homopolymer of approximately 18 perosamine residues which are substituted with tetronate (30). Recently, the structure of the LPS core oligosaccharide

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was reported for O1 and O139 mutants (33, 64); the results from these investigators are summarized in Fig. 1A. The site of attachment for the O1 antigen is unknown, whereas the O139 attachment for the O1 antigen is based on the structural analysis of Knirel et al. (33) and Vinogradov et al. (64), indicating a conserved core oligosaccharide for both serogroups. The site of the attachment of the O1 antigen has not been determined. The O139 antigen was found to be attached to the HepIII residue. Hep, 1-glycero-D-manno-heptose; Kdo, 3-deoxy-D-manno-octulosonic acid; PEmn, 2-aminoethyl phosphate. (B) Analysis of LPS by SDS-PAGE and Silver staining. Lanes: 1, P27459res29/pACYC177; 2, P27459res29/pACYCgalU; 3, P27459galU; 4, P27459ΔgalE::cm; wt, P27459.

Materials and Methods

Bacterial strains, plasmids, and media. V. cholerae strains used in this study are listed in Table 1. Escherichia coli K-12 strain LE392 (54) was used for all genetic constructions, unless the vector being used was a derivative of pCVD442 (13); in this case E. coli SM10Xpir (42) was used. All strains were grown in Luria broth (LB) at 37°C except as noted otherwise. Antibiotics were used at the following concentrations: kanamycin, 50 μg/ml; and chloramphenicol, 2.5 μg/ml (V. cholerae) or 30 μg/ml (E. coli); ampicillin, 30 or 100 μg/ml; and streptomycin, 100 μg/ml. Plasmids used in this study are listed in Table 1.

Oligonucleotides, PCR, and Southern hybridization. All oligonucleotides used for PCR are listed in Table 2. PCRs were performed as described by Mullis and Faloona (44). Hybridization with horseradish peroxidase-labeled probe and detection of hybridizing bands were carried out according to the procedure provided by the manufacturer of the ECL (enhanced chemiluminescence) system (Amersham Pharmacia Biotech, Freiburg, Germany). Hybridization with horseradish peroxidase-labeled probe and detection of hybridizing bands were carried out according to the procedure provided by the manufacturer of the ECL (enhanced chemiluminescence) system (Amersham Pharmacia Biotech, Freiburg, Germany).

Computer analysis. The publicly available sequences from the V. cholerae genome project (http://www.tigr.org) (22) were examined for the presence of gal genes. The amino acid sequences of E. coli GalE and GalU, obtained from the database, were used as query sequences. Homology analysis was performed with Basic BLAST Search 2.0 (1).

Construction of plasmids. To construct complementing plasmids, gene-specific oligonucleotides (Table 2) were designed to introduce PirI and FspI sites at the 5’ and 3’ ends, respectively, of the genes galU and galE. Following PCR amplification, the products were digested and ligated into the PirI- and FspI-digested plasmid pACYC177. The resulting plasmids pACYGalU and pACYCgalE express the corresponding genes from the bla promoter. To construct suicide vector pCVDgalU, an internal fragment of galU, obtained after PCR with primers galU1 and -2, was ligated into the SacI/XbaI-digested plasmid pCVD442 (13); this plasmid was used to construct an insertion mutation in the galU gene. Suicide phorylase, encoded by galU, and UDP-glucose-4-epimerase, encoded by galE (36). In some bacteria, GalE and GalU are also important in the catabolism of exogenous galactose together with enzymes encoded by galT and galK (36). In many gram-negative pathogens, mutations in galE or galU lead to attenuated virulence, mainly if changes in LPS or capsular structures were observed (18). However, nothing is known about the role of galU and galE in V. cholerae O1 virulence.

Recently, temperate phage K139 (51) was found to use the O antigen of V. cholerae O1 as its receptor (46). A collection of spontaneous phage-resistant O1 El Tor P27459 strains were investigated according to their LPS pattern on silver-stained polyacrylamide (PAA) gels, and different groups of altered LPS structures were described. These included O-antigen-negative strains, where one mutant was characterized for the loss of O antigen due to transposition of IS1004 into wbeW, encoding a putative glycosyltransferase. Another class identified were rough LPS (R-LPS) mutants comprising an altered core oligosaccharide with no O antigen attached; however, the mutation(s) responsible remains unidentified. In this study, we investigated the genetic nature of a phage-resistant mutant with intact O antigen but altered core oligosaccharide composition and found that this strain contained a mutation in galU. We also compared galU with galE and LPS-defective (R-LPS and O-antigen-negative) mutants with regard to LPS synthesis, galactose catabolism, the ability to colonize the small intestine of mice, and the ability to survive in the presence of bactericidal substances. Among our spontaneous phage-resistant mutants, we also found rugose colony variants (unpublished results). In this work, we provide evidence that galU and galE are involved in rugose polysaccharide production.
plasmids to introduce chromosomal in-frame deletions of the genes *wbeW* and *galE* were constructed in the same manner. DNA fragments of about 500 bp encompassing the regions upstream of the corresponding gene were PCR amplified using primer pairs 5′-X and ligated together with the 3′-SmaI fragment. Strains harboring the chloramphenicol-resistant (Cm r) plasmid pKEK-PCR-derived cat SmaI into wild type (O1 El Tor, Inaba), spontaneous Smc.

The downstream DNA region of the corresponding gene was PCR amplified using primer pairs 5′-galE and ligated overnight (O/N). The ligation mix was digested with SmaI and ligated into pJN229 pCVD442 with alternative MCS; A p r 9

To construct strains containing a mutation in *wbeW* plasmid pKEK229 pCVD442 with alternative MCS; A p r 13

Strains

Plasmids

To give the chloramphenicol-resistant (Cm r) plasmid pKEK-PCR-derived cat SmaI and ligated overnight (O/N). The ligation mix was digested with SmaI and ligated into pJN229 pCVD442 with alternative MCS; A p r 13

Strains

Plasmids

Table 1. *V. cholerae* strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or description</th>
<th>Source or reference</th>
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<td>CO970</td>
<td>O1 El Tor Ogawa, India 1994, Smc Cm'</td>
<td>This study</td>
</tr>
<tr>
<td>CO970galU</td>
<td>galU::P::CVD442, Ap'</td>
<td>This study</td>
</tr>
<tr>
<td>O395CTX-Km</td>
<td>Km'</td>
<td>This study</td>
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<tr>
<td>P27459-S</td>
<td>Wild type (O1 El Tor, Inaba), spontaneous Smc'</td>
<td>This study</td>
</tr>
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<td>P27459galE::cm</td>
<td>Smc' in P27459-S, Cm'</td>
<td>This study</td>
</tr>
<tr>
<td>P27459galU</td>
<td>galU::P::CVD442 in P27459-S, Ap'</td>
<td>This study</td>
</tr>
<tr>
<td>P27459galZ</td>
<td>lacZ::PMu13 in P27459-S, Ap'</td>
<td>This study</td>
</tr>
<tr>
<td>P27459res29</td>
<td>Altered core oligosaccharide, intact O antigens; mutant of P27459-S</td>
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</tr>
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<td>P27459res46</td>
<td>R-LPS mutant of P27459-S</td>
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<tr>
<td>P27459res105</td>
<td>RUGose colony variant of P27459-S</td>
<td>This study</td>
</tr>
<tr>
<td>P27459res105galE::cm</td>
<td>Smc' in P27459res105, Cm'</td>
<td>This study</td>
</tr>
<tr>
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<td>P27459galU wbeW</td>
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<tr>
<td>PTr49k</td>
<td>Km'</td>
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Table 2. Oligonucleotides used in this study

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<tr>
<td>cm2</td>
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</tr>
<tr>
<td>galEps1</td>
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<td>5′galEsa1</td>
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<td>3′galU</td>
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<tr>
<td>3′galU2</td>
<td>GATCGTCTAGTTGTGTTAGATGACAGAAG</td>
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</tbody>
</table>

Underlined nucleotides are not exact matches to the sequence and were introduced to add restriction enzyme sites. Nucleotide sequences were derived from the publicly available sequences from the *V. cholerae* genome project (http://www.tigr.org) (22) and accession no. X59954 and Y07788.

Serum resistance assay. Cells were grown to mid-logarithmic phase in LB, washed, and mixed to a final concentration of 20% with normal human serum (obtained and pooled from four donors) or 20% heat-inactivated normal human serum in phosphate-buffered saline with 0.1% peptone. Approximately 10^6 CFU was used in each assay. After incubation at 37°C for 1 h, the cells were harvested, washed, and resuspended in phosphate-buffered saline with 0.1% peptone. Vi- able cell counts were determined by plating serial dilutions on LB agar.

Mouse colonization assays. The infant mouse colonization assay has been described previously (32). Briefly, mutant strains (lac') were mixed with the strain P27459lacZ (lac') and given in a peroral inoculum ratio of approximately 10^6 CFU mutant to 10^7 CFU of wild type to 5- to 6-day-old CD-1 suckling mice. After a 24-h period of colonization, intestinal homogenates were collected and the ratio of mutant to wild type was determined by plating appropriate dilutions on LB agar containing streptomycin and X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside).

CTX-Km® transduction and Gm®-ELISA. For optimal CT and TCP expression, the strains were incubated under inducing conditions. O/N cultures grown in AKI medium were diluted 1:100 in AKI without NaHCO₃, grown for 4 h at 37°C under static growth conditions, and then transferred for vigorous shaking for 4 h at 37°C (40). For CT measurement, 1 ml of the culture was spun down, and the culture supernatant was filtered (0.2-μm-pore-size filter; Schleicher & Schuell, Dassel, Germany), and frozen at −20°C. The level of CT present in 200 μl of the supernatant was measured by Gm®-ganglioside enzyme-linked immuno- nosorbent assay (Gm®-ELISA) (61) using purified CT (Sigma, Deisenhofen, Germany) to generate a standard curve.

For phage transduction, 100 μl of the same culture was mixed with 100 μl of phage CTX-Km® (66). After standing for 30 min at room temperature, the mixture was diluted and plated onto LB agar containing kanamycin. The transduction frequency was calculated from the CFU of kanamycin-resistant (Km r) transductants correlated with the optical density at 600 nm (OD₆₀₀) of the recipient strain. An additional CTX-Km® transduction experiment was performed to enumerate the numbers of both transductants and potential recipients. Strain P27459ΔreHr was used as a negative control for both assays. The ΔtoxI mutation was introduced into the chromosome of P27459-S by plasmid pMD60, as previously described (31), to form strain P27459ΔreHr. Phage CTX-Km® was prepared from a 100-ml O/N culture (LB, 30°C) of strain O395CTX-Km by filtering the culture supernatant through a 0.2-μm-pore-size filter.

Determination of MICs of antimicrobial cationic peptides. Standard MIC testing of susceptibility to polymyxin B (50 μg/ml to 780 ng/ml; Sigma) and protamine (675 to 10.5 μg/ml; Sigma) was done as described by Steinberg et al. (57). The MIC was defined as the lowest concentration of drug which prevented visible turbidity after incubation for 18 h at 37°C without shaking.

Sensitivity to hydrophobic agents. Sensitivity to SDS and novobiocin was assessed by determination of the MIC. O/N cultures of strains to be tested were diluted in LB to give 1 × 10⁵ to 5 × 10⁶ CFU/ml. After 100-μl aliquots of the diluted cultures were dispensed into each well of a 96-well microtiter plate (Greiner, Frickenhausen, Germany), 1 μl of test compound was added. SDS (Merck, Hamburg, Germany) and novobiocin (Sigma) were dissolved in water at

Underlined nucleotides are not exact matches to the sequence and were introduced to add restriction enzyme sites. Nucleotide sequences were derived from the publicly available sequences from the *V. cholerae* genome project (http://www.tigr.org) (22) and accession no. X59954 and Y07788.
10-fold the desired test concentration and then 2-fold serially diluted in water. The final concentrations tested ranged from 600 to 9.3 μg/ml for SDS and 1.5 μg/ml to 23 ng/ml for novobiocin. The sensitivity to bile was tested on thiosulfate citrate bile sucrose (TCBS) agar plates, prepared as instructed for the commercially available TCBS (Difco, Heidelberg), containing various amounts of bile (0 to 0.8%).

Bioluminescence measurement. Quantitative bioluminescence measurement was performed in a microtiter assay as described previously (50), with minor modifications. Briefly, bacteria were grown O/N in LB and diluted 1:100 in fresh LB containing appropriate antibiotics; then 100-μl aliquots were placed in a 96-well microtiter plate (Costar; Greiner) and incubated for 24 h at room temperature. Bacterial cultures were poured out, washed three times with H2O, fixed with 2.5% glutaraldehyde, washed twice, and stained with 200 μl of a 0.4% crystal violet solution. After solubilization of the crystal violet with 300 μl of ethanol-acetone (80:20), the OD was determined at 570 nm in an ELISA reader (Bio-Rad, Munich, Germany). Qualitative bioluminescence formation was measured by incubating 1:100-diluted O/N cultures (LB, with appropriate antibiotics) in borosilicate tubes for 48 h without shaking at room temperature. Strains were noted as bioluminescent positive if the OD was determined at 570 nm in an ELISA reader (Bio-Rad, Munich, Germany).

Plaque inhibition assay. The phage neutralizing capacity of purified LPS was determined as described previously (46).

β-Lactamase detection by immunoblotting analysis. Cells were harvested at OD600 1.5 to 2.5 and diluted to give 2 ml of OD600 = 1. After centrifugation of 1 ml of cell suspension, the cell pellets were resuspended in 100 μl of sample buffer. The supernatants were filtered, and proteins were precipitated with 10% trichloroacetic acid. The protein pellet was solubilized in 100 μl of sample buffer. Samples (20 μl) were separated by SDS-PAGE (34), transferred to a nitrocellulose membrane, and reacted with an antibody directed against BlaM (5 Prime Inc., Boulder, Colo.).

RESULTS

Identification of a spontaneous galU mutant expressing an altered LPS core oligosaccharide. The recently described spontaneous phage K139,cm9-resistant mutant P27459res29 showed an altered LPS core oligosaccharide with an intact O antigen (Fig. 1B, lane 1) (46). The observation that this strain could not grow on M9 medium containing galactose as a carbon source and the fact that glucose is a component of the LPS biosynthesis. Knockout constructions in introduced into strain P27459-S are illustrated schematically (Materials and Methods).

enterica serotype Typhimurium, the genetic loss of galE and galU causes bacteriolysis when growing cells are exposed to high concentrations of r-galactose (36). This property was tested in V. cholerae galE and galU mutants on LB plates containing 2% galactose. The galE mutant could not grow on such plates, whereas both galU mutants (spontaneous and constructed) grew initially but eventually lysed. This phenotype could be complemented by introduction of galU or galE in trans. The LPS patterns of both galE and galU mutants were then analyzed by SDS-PAGE. In a silver-stained PAA gel, the galU mutant showed an altered core with an intact O antigen (Fig. 1B, lane 3). In contrast, the LPS of the galE mutant migrated similarly to the LPS of the wild-type (wt) strain (Fig. 1B, lane 4), revealing that GalE does not affect LPS biosynthesis. Another evidence for the presence of intact LPS, the finding that the galE mutant is as sensitive as the wt strain in cross-streak analysis versus phage K139,cm9 (data not shown).

Colonization of gal and LPS mutants in infant mice. The ability of gal mutants to colonize the small intestine was assessed in a competition assay using perorally infected CD-1 suckling mice. To compare the colonization behaviors of the gal mutants and other LPS mutants, we also investigated the spontaneous R-LPS mutant P27459res46 and a defined O-antigen-negative strain (ΔwbeW; Materials and Methods). As shown in Table 3, strain P27459ΔwbeW was significantly reduced in its ability to colonize the mouse small intestine, a result also found for other O-antigen-deficient V. cholerae strains (gmd [67] and manB [6]). No mutant bacteria were recovered from mice inoculated with the R-LPS mutant P27459res46, harboring an altered LPS core oligosaccharide without attached O antigen. The core oligosaccharide-defective galU mutants (P27459 res29, P27459 galU) were approximately 50- to 100-fold attenuated in the ability to colonize, although they carry the O antigen. The colonization defect of the spontaneous mutant P27459res29 was fully complemented by the presence of a plasmid expressing galU, revealing that the colonization defect of this strain is due to a mutation in galU. Importantly, the galE mutant with intact LPS colonized as well as the wt strain, indicating that the colonization defect of galU-deficient strains is due to neither their galactose sensitivity nor their defect in utilizing exogenous galactose. These

FIG. 2. Chromosomal organization of investigated gal genes and surrounding ORFs. While this report was under review, the complete genomic sequence of V. cholerae was published (22). The representation is therefore based on data from the V. cholerae genomic database (http://www.tigr.org). galE (VCA0773) is located on chromosome 2, whereas galU (VC0395) is located on chromosome 1. Based on homology analysis, the surrounding ORFs seem not to be involved in galactose-catabolism or LPS biosynthesis. Knockout constructions introduced into strain P27459-S are illustrated schematically (Materials and Methods).
results suggest that the core oligosaccharide defect in galU mutants attenuates *V. cholerae* intestinal colonization.

**Outer membrane integrity of LPS mutants.** Misassembly of outer membrane protein multimers in LPS mutants, and the significance of LPS to transporters, has been described for some gram-negative bacteria (35, 68). In *V. cholerae*, the presence of the type IV pilus TCP during infection is important for colonization (62). We measured TCP expression by a transduction assay using CTX-KmΦ, a Km' version of phage CTXΦ, which is known to use TCP as its receptor (66). As indicated in Table 3, there were no significant differences in the frequency of phage transduction in galU, wbeW, and R-LPS strains. These data indicated that there is functional TCP on the surface of all three LPS-deficient mutants, allowing at least an efficient phage CTXΦ transduction. In addition, we investigated the presence of the main virulence factor CT and hemagglutinin-protease (HAP) in the extracellular medium, since both enzymes are known to be secreted by the type II secretion apparatus eps (extracellular protein secretion) (53). CT concentration in the supernatant was measured by *G. stearothermophilus* ELISA, and HAP activity was determined on agar plates containing 1% nonfat milk agar plates. As shown in Table 3, no significant differences were found between the LPS mutants and the wt strain.

Outer membrane integrity was further studied by investigating the localization of the periplasmic enzyme β-lactamase, expressed from plasmid pBR322, in Western blot analysis. Very little β-lactamase was detected in culture supernatants of the wt strain, (Fig. 3B, lane 2), the galU mutant exhibited slightly more (lane 3), and the spontaneous R-LPS mutant produced enhanced amounts (lane 4). The latter observation correlates with less β-lactamase in corresponding whole-cell extracts (Fig. 3A, lane 4). However, no significant difference of β-lactamase production was observed between wt and the galU mutant in whole-cell extracts (Fig. 3A, lane 2 and 3). We have found no evidence that the increased level of β-lactamase in the supernatant is due to enhanced cell lysis (e.g., by observing normal growth abilities in liquid cultures and the inability to detect activity of the cytoplasmic enzyme LacZ in the supernatants [data not shown]). The results suggest that the core-deficient mutants are leaky for periplasmic proteins, enhanced for the R-LPS and less leaky for the galU mutant.

**Survival in the presence of organic acids.** *V. cholerae* may be exposed to high concentrations of weak organic short-chain acids present in the gastrointestinal tracts of humans (12). It is well known that weak organic acids can permeate the cytoplasmic membrane in the protonated form (depending on environmental pH), deprotonate, and accumulate intracellularly in the harmful anionic form (17). We investigated the survival of the three different types of LPS mutants after exposure to short-chain acids. The O-antigen mutant (ΔwbeW) survived after exposure for 1 h to the shock medium (LB containing 2.5 mM butyric acid, 8.7 mM acetic acid, and 3.7 mM propionic acid [pH 4.4]) as well as the wt strain (Table 4). In contrast, mutant strains with an incomplete LPS core oligosaccharide are apparently more susceptible to organic acids. The galU mutant (res29) was about 75-fold attenuated, while the R-LPS mutant (res118) showed the most severe defect. Merrell and Camilli (41) reported that *V. cholerae* is capable of mounting an acid tolerance response which protects against such substances. Therefore, we tested the survival of the LPS core oligosaccharide mutants after adaptation for 1 h in LB (pH 5.7) before placement in the shock medium. All mutants survived as well as the wt strain after a 1-h exposure, but after longer exposure (>2 h), the R-LPS mutant was approximately 50-fold attenuated (data not shown). These results suggest that an intact LPS core region, in addition to the inducible acid tolerance response, provides protection against protonated weak acids.

**Sensitivity to bile and other hydrophobic agents.** The LPS core oligosaccharide region of gram-negative bacteria appears to act as an effective outer membrane barrier against hydrophobic agents with charged groups (47). To determine whether this is also true for *V. cholerae*, we tested growth on TCBS plates containing 0.8% bile. In the human intestine, the concentration of bile salts is estimated to be ~1% (depending on nutrition status) (24). As shown in Fig. 4, galU (res29) and R-LPS (res118) mutants did not grow on these plates. However, the R-LPS mutant also grew poorly on the same plates.

### Table 3. Intestinal colonization, CTX-KmΦ phage transduction, and CT release

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mouse CT'</th>
<th>CTX-KmΦ transduction</th>
<th>CT pro-duction</th>
<th>HAP secretion</th>
</tr>
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<tbody>
<tr>
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<td>+</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
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<td>+</td>
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<td>1.94 (5)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>P27459galU res46/wbeW</td>
<td>0.96 (6)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P27459wbeW</td>
<td>0.024 (5)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P27459wbeW/lac</td>
<td>0.8 (4)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>P27459res29/lac</td>
<td>&lt;10⁻¹⁵ (5)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* The competitive index (CI) for colonization is defined as the output ratio of mutant to wt bacteria divided by the input ratio of mutant to wt bacteria (i.e., Strains P27459galU, P27459res29, P27459res29/pACYC177, P27459wbeW, and P27459res46 had significant differences in colonization compared to the wt strain (P27459) as determined by Student's two-tailed t test (P < 0.01); all other strains showed less significant differences (P > 0.01). In vitro competition assays were also done for galU mutants: P27459res29, CI 1.7; P27459res29/pACYC177, CI 4.7; P27459galU, CI 1. The in vitro CIs demonstrate that galU mutants had no handicap or growth defect versus the wt under in vitro conditions.

* Determined in triplicate (Materials and Methods). The frequency of transduction ranged from 10⁻¹ to 10⁻³, with no significant differences between the strains tested.

* Measured in duplicate (Materials and Methods). The levels of CT present in the culture supernatants were between 0.5 and 2 μg ml⁻¹ OD₆₀₀, with no significant differences between the strains tested.

* Detected as a zone of clearing on 1% nonfat milk agar plates.

* ND, not determined.

* Mouse colonization assays were initially performed with strain P27459res46, an R-LPS mutant which lacks O antigen and has an altered core, like P27459res118. All other experiments were done with strain P27459res118. To preserve mice, we did not repeat the colonization assays with strain P27459res118.

**FIG. 3.** Effect of LPS alteration on the localization of β-lactamase. BlaM was immunodetected by ECL as indicated in Materials and Methods. (A) Whole-cell extracts; (B) supernatant extraction. Lanes: 1, wt P27459 without plasmid; 2, wt P27459/pBR322; 3, spontaneous galU mutant SP27459/res29/pBR322; 4, R-LPS mutant SP27459/res118/pBR322. The experiment was performed in duplicate with independently growing cells. Samples were matched by equivalent OD₆₀₀ units.
lacking bile, while the \textit{galU} mutant grew similar to the wt strain. \textit{O}-antigen-negative (\textit{wbeW}) mutants, \textit{galE} mutants, and \textit{galU} mutants complemented with a \textit{galU} expression plasmid were able to grow on TCBS containing bile. A growth defect of \textit{galU} and R-LPS mutants was also observed in LB containing >0.1% of the bile component deoxycholate (data not shown). In addition, the sensitivity of the \textit{galU} and R-LPS mutant strains to anionic hydrophobic agents was confirmed by determining the MICs of SDS and novobiocin. As indicated in Table 4, sensitivity to these peptides decreased in the following order: R-LPS mutant (\textit{res118}) > \textit{galU} mutants (\textit{res29}, \textit{galU}) > \textit{O}-antigen-negative strain (\textit{\textDelta wbeW}) > wt = \textit{galE} mutant = \textit{galU} mutants containing plasmid pACYCgalU. These results indicate that the LPS core oligosaccharide is more important than the \textit{O}-antigen for resistance against cationic peptides.

Recently, it was demonstrated that \textit{V. cholerae} \textit{O}-antigen-negative strains are sensitive to complement activity (5, 67), as determined by serum resistance assays. To analyze whether the LPS core oligosaccharide also contributes to serum resistance, the \textit{galU} mutants (\textit{res29}, \textit{galU}) were investigated in a serum bactericidal assay (Materials and Methods). \textit{galU} mutants exhibited markedly greater serum sensitivity than wt or \textit{galE} strains (Table 4). Survival was restored in \textit{galU} mutants harboring complementation plasmid pACYCgalU. Strains lacking the \textit{O} antigen (\textit{\textDelta wbeW}, R-LPS) were about 100-fold more sensitive to human serum than \textit{galU} mutants, indicating that while an intact core oligosaccharide plays a critical role, the \textit{O} antigen is the most important factor in complement resistance.

**Role of \textit{galE} and \textit{galU} in biofilm formation.** Among our spontaneous phage K139.cm9-resistant P27459 isolates, we found strains with a rugose colony morphology. Such variants have been described previously (72) to be constitutively synthesizing an exopolysaccharide (VPS) allowing the bacteria to

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**TABLE 4. Resistance against organic acids, hydrophobic agents, antimicrobial peptides, and the complement**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Resistance to organic acids (%) survival</th>
<th>MIC ((\mu g/ml))a</th>
<th>Serum resistance (%) survival</th>
<th>Strain</th>
<th>Resistance to organic acids (%) survival</th>
<th>MIC ((\mu g/ml))a</th>
<th>Serum resistance (%) survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SDS</td>
<td>Novobiocin</td>
<td>Polymyxin B</td>
<td>Protamine</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>P27459-S</td>
<td>5.7 (\times) 10^{-3} \pm 2.0 (\times) 10^{-3}</td>
<td>300</td>
<td>0.75</td>
<td>12.5</td>
<td>337</td>
<td>85 (\pm) 15 (5)</td>
<td></td>
</tr>
<tr>
<td>P27459galU</td>
<td>NDa</td>
<td>150</td>
<td>0.187</td>
<td>1.56</td>
<td>84</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>P27459galU/TrcAkan</td>
<td>ND</td>
<td>150</td>
<td>0.187</td>
<td>ND</td>
<td>ND</td>
<td>0.21 (\pm) 1 (1)</td>
<td></td>
</tr>
<tr>
<td>P27459galU/pACYCgalU</td>
<td>ND</td>
<td>300</td>
<td>0.75</td>
<td>12.5</td>
<td>337</td>
<td>100 (3)</td>
<td></td>
</tr>
<tr>
<td>P27459res29</td>
<td>7.6 (\times) 10^{-5} \pm 8.9 (\times) 10^{-5}</td>
<td>150</td>
<td>0.187</td>
<td>1.56</td>
<td>84</td>
<td>0.6 (\pm) 1 (4)</td>
<td></td>
</tr>
<tr>
<td>P27459res29/pACYC177</td>
<td>ND</td>
<td>150</td>
<td>0.187</td>
<td>ND</td>
<td>ND</td>
<td>1.1 (\pm) 1.6 (2)</td>
<td></td>
</tr>
<tr>
<td>P27459galE::con</td>
<td>ND</td>
<td>300</td>
<td>0.75</td>
<td>12.5</td>
<td>337</td>
<td>100 (3)</td>
<td></td>
</tr>
<tr>
<td>P27459galU::galE::con</td>
<td>ND</td>
<td>300</td>
<td>0.75</td>
<td>12.5</td>
<td>337</td>
<td>81 (\pm) 26 (2)</td>
<td></td>
</tr>
<tr>
<td>P27459\Delta wbeW</td>
<td>5.8 (\times) 10^{-3} \pm 1.7 (\times) 10^{-3}</td>
<td>300</td>
<td>0.75</td>
<td>6.25</td>
<td>168</td>
<td>0.006 (\pm) 0.0056 (2)</td>
<td></td>
</tr>
<tr>
<td>P27459\Delta wbeW/pACYC177</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>6.25</td>
<td>168</td>
<td>0.001 (\pm) 0.0019 (2)</td>
<td></td>
</tr>
<tr>
<td>P27459\Delta wbeW/pJNwbeW</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>12.5</td>
<td>337</td>
<td>100 (2)</td>
<td></td>
</tr>
<tr>
<td>P27459galU::res118</td>
<td>0</td>
<td>150</td>
<td>0.047</td>
<td>0.78</td>
<td>21</td>
<td>0.0003 (\pm) 0.0004 (2)</td>
<td></td>
</tr>
</tbody>
</table>

a Survival of wt and mutant strains in response to short-chain organic acid challenge. Cells were grown in LB (pH 7) and subsequently shocked in LB (pH 4.4) containing 8.7 mM acetic acid, 2.5 mM butyric acid, and 3.7 mM propionic acid. Approximately 10^6 CFU/ml was used in each experiment. Percent survival at 60 min was calculated by comparison with the initial number of cells measured at time zero. Data shown are the average from triplicate experiments \(\pm\) standard deviation.

b All experiments were performed in triplicate.

c Calculated as 100 \(\times\) (number of CFU after cells were incubated for 1 h in 20% normal human serum/number of CFU after cells were incubated for 1 h in 20% heat-inactivated normal human serum). Indicated is the mean \(\pm\) standard deviation. The numbers of independent experiments are given in parentheses.

d ND, not determined.
form a biofilm on abiotic surfaces. The composition of the VPS from two different V. cholerae O1 El Tor rugose isolates has recently been published. Wai et al. found a VPS consisting of N-acetyl-D-glucosamine, N-mannose, 6-deoxy-D-galactose, and O-galactose (65), whereas Yildiz and Schoolnik described the composition of the VPS as glucose, galactose, N-acetylglucosamine, mannose, and xylose (72). These findings prompted us to investigate a possible role of galE and galU in production of the VPS.

The parental wt strain P27459 was unable to form a biofilm on polystyrene or borosilicate glass, in contrast to the rugose variant P27459res105. To study the influence of galU and galE mutations on biofilm formation, we introduced these mutations into the rugose variant P27459res105. Both mutations yielded smooth colony forms, suggesting that galU and galE mutants are unable to synthesize the VPS. Introducing plasmids carrying galU or galE restored the rugose colony morphology. The phenotypes were confirmed in a quantitative biofilm measurement assay (Fig. 5A). To confirm the galU phenotype in a nonrugose wt V. cholerae O1 El Tor strain, we screened several clinical isolates for the ability to produce a biofilm in polystyrene microtiter dishes. Strain CO970, which formed a biofilm, was chosen for further investigation. As shown in Fig. 5B, a galU mutant of strain CO970 was no longer able to form a biofilm on borosilicate glass tubes. Our results indicate that UDP-glucose or UDP-galactose is necessary for biosynthesis of the VPS.

**Rugose phenotype and phage K139 accessibility.** We also wished to determine why spontaneous rugose variants of strain P27459 are phage resistant. Since the O antigen is the receptor of phage K139, we first analyzed the LPS structure of rugose strain P27459res105 by SDS-PAGE analysis (silver staining and Western blotting). Furthermore, the ability of the phage to bind to purified LPS from P27459res105 was determined in a plaque inhibition assay (46). Neither type of analysis revealed differences from the wt LPS, indicating that the phage could potentially bind to strain P27459res105 LPS. Second, we investigated the sensitivity to phage K139 of the nonrugose mutant P27459res105ΔgalE::cm in cross-streaking and plaque formation experiments. The nonrugose mutant is phage sensitive, in contrast to its parent rugose variant (data not shown). All results together provide evidence that production of the VPS limits the access of phage K139 to its receptor, the O antigen, hence leading to attenuated phage susceptibility.

**DISCUSSION**

In this work, we identified a mutation in galU that caused the spontaneous bacteriophage K139.cm9-resistant phenotype of strain P27459res29. The O antigen of galU mutants is able to be adsorbed by phage K139; nevertheless, these strains are phage resistant (46). The mechanism responsible for this phenotype is unresolved. The structure of the core oligosaccharide in galU-deficient strains is not known; however, preliminary evidence suggests that it contains no glucose. We predict that galU-deficient strains lack the α-Glc and β-Fru-β-Glc branches on the HeP1 residue (Fig. 1A), but proof of this awaits structural analysis.

We demonstrated that galU mutants were significantly reduced in the ability to colonize the mouse small intestine. This phenotype could be caused by a pleiotropic effect, because galU mutants are predicted to be unable to synthesize UDP-glucose. Besides its function as a substrate for glucosyltransferases resulting in glucosylated surface structures, UDP-glucose plays a well-established biochemical role as a glycosyl donor in the enzymatic biosynthesis of carbohydrates. Some examples in gram-negative bacteria are synthesis of the osmoprotectant trehalose (under conditions of high osmolarity) and membrane-derived oligosaccharide (under conditions of low osmolarity) in E. coli (11); synthesis of UDP-arabinose, which is incorporated into lipid A of S. enterica serovar Typhimurium to protect against antimicrobial cationic peptides, especially polymyxin B (14); and synthesis of UDP-galactose, which serves as a donor for several surface structures, including glycosylated pili in Neisseria meningitidis (58). It is less clear whether UDP-glucose additionally acts as an intracellular signal molecule. For one study, it was reported that UDP-glucose controls the expression of σs in E. coli (4).

Investigating both galU and galE mutants, we ruled out the possibility that the observed colonization defect of V. cholerae O1 galU mutants is due to a conferred galactose sensitivity or to the inability to catabolize galactose, both of which are phenotypes of galE and galU mutants (Fig. 6). Unlike the galU strain, the galE mutant was found to colonize the mouse small intestine as well as the wt strain. The most apparent difference
between the galE and galU mutants lies in their LPS structures. galE-deficient strains show an apparently normal LPS, while galU-deficient strains have altered core oligosaccharides. Two observations do not support the possibility that UDP-galactose, synthesized from nontoxic concentrations of exogenous galactose in galE mutants, could be incorporated into the LPS, as known for other gram-negative bacteria (38). First, compositional and structural analyses of O1 LPS have failed to detect galactose (29, 30, 64). Second, a V. cholerae O1 galEK double mutant, insensitive to galactose and unable to synthesize UDP-galactose from exogenous galactose (Fig. 6), apparently produced wt LPS as determined by SDS-PAGE analysis (silver staining and Western blotting) and was able to colonize the small intestines of mice similar to a wt strain (unpublished results).

According to the V. cholerae genome sequences (22), open reading frame (ORF) VC0262, located within the rbh gene cluster, is annotated as a second galE gene. However, in contrast to the galE gene investigated in this study (VCA0774; 65.4% identity to galE of E. coli in 338 amino acids), ORF VC0262 shows only low homology to the galE gene of E. coli (26.6% identity in 169 amino acids). In addition, Fallarino et al. (15) suggested that ORF VC0262 is unlikely a galE gene, showing that it could not complement an S. enterica serovar Typhimurium galE mutant. Furthermore, they found that V. cholerae mutants within ORF VC0262 synthesize a normal LPS. Taking the data together, we conclude that our constructed galEK double mutant could not synthesize UDP-galactose at all, which suggests that no galactose-containing surface structures in V. cholerae O1 El Tor are involved in colonization of the small intestine in mice.

One explanation for the colonization defect of galU mutants is that alteration of the LPS core region reduced their ability to survive within the intestine. To address the role of an intact LPS core oligosaccharide in V. cholerae intestinal survival, we tested the effects of several substances known to be present in the human intestine. Three different types of LPS mutants were compared: one lacking the O antigen (wbeW), one with an altered core (galU), and one lacking an O antigen and expressing an altered core (R-LPS). These comparative studies demonstrated several functions of the V. cholerae LPS substructures. An intact core oligosaccharide, without attached O antigen, is sufficient for survival in the presence of anionic detergents (e.g., bile salts) and short organic acids. However, effective protection against these substances is no longer provided by galU and R-LPS mutants. These results suggest that an altered core oligosaccharide increases the susceptibility to short protonated organic acids and anionic detergents. The observed growth inhibition seen in anionic detergent MIC assays is probably due to the disruption of the inner membrane integrity. Enteric bacteria like E. coli are known to need additional efflux pumps for sufficient protection against hydrophobic substances such as bile salts (73), and recently one such system has been identified in V. cholerae (7).

Both V. cholerae LPS substructures, core and O antigen, were found to have roles in resistance to bactericidal substances of the innate immune system. The core oligosaccharide region was found to be more important than the O antigen in resisting the outer membrane-disturbing action of cationic peptides. Resistance against cationic peptides can depend on several factors, such as the charge of the LPS molecules or LPS concentration in the membrane. In addition, it is known that some gram-negative bacteria are capable of activating an inducible resistance mechanism, such as the modification of lipid A and core oligosaccharides with aminoarabinose and ethanolamine, which reduces the negative charge of inner anionic regions and thereby reduces binding of the cationic peptides (14). It is not known if V. cholerae induces modifications of lipid A; hence, the sensitivity of galU mutants to cationic peptides such as polymyxin B could also be due to an inability to synthesize aminoarabinose from UDP-glucose (see above). Interestingly, the most important LPS structure for resistance against the complement system is the O antigen (67) rather than the core LPS. However, our experiments demonstrated that for effective protection against complement components, an intact core oligosaccharide is also necessary.

The results of the in vitro assays suggest that in galU mutants the integrity of the outer membrane is compromised and it no longer represents an effective barrier. The instability of the outer membrane in galU mutants could be due to weakened LPS-core-LPS core interactions. Disruption or alterations of the outer membrane integrity could eventually cause enhanced penetration of proteins and diffusion of periplasmic material, resulting in periplasmic leakage (47, 63). Evidence for the loss of periplasmic proteins of a galU mutant strain was supported by the observation that β-lactamase, but not the cytoplasmic enzyme β-galactosidase (data not shown), was found in slightly increased amounts in the supernatant. This may suggest that active HAP and CT, normally secreted via the eps system, could also escape from the periplasm due to penetration or diffusion across the outer membrane. However, other than slightly increased β-lactamase in supernatants and sensitivity against hydrophobic substances, we found no evidence for outer membrane damage, since outer membrane preparations of galU strains indicated no visible protein alterations compared to the wt strain (data not shown). Additionally, we found TCP, which could serve as phage receptor for CTXΦ phage transduction. Since TCP is also required for colonization of the small intestine, we suggest that the colonization defect of the
galU mutants is not due to alterations of TCP. However, it could also be possible that the quantity or function of TCP is altered in the mutant strain within the harsh environment of the small intestine. The secretion of virulence factors and the assembly of TCP in galU mutants remain to be addressed.

The spontaneous R-LPS mutants showed the most severe defect in colonization and survival in in vitro assays. The mutation in strain P27459res118 remains unidentified, but we speculate that it is affected in the HepII-transferase (Fig. 1A), since it has been hypothesized that V. cholerae O1 heptoseless mutants are nonviable (60). The alteration in the core oligosaccharide in this R-LPS mutant (res118) was found to have a more drastic effect on outer membrane integrity. It was expected that this type of mutant could not colonize the small intestine because it lacks the O antigen. The exact role of the LPS O antigen in colonization is unknown. An early report hypothesized that a lack of the pilus TCP leads to attenuated colonization (26); however, recently it was reported that O-antigen-negative strains express TCP (5). We can also demonstrate that TCP function as the CTXφ phage receptor is maintained in the O-antigen-negative strains, which is consistent with the finding of Chiang and Mekalanos (5). One known phenotype of O-antigen mutants is a remarkable sensitivity against the complement system (67). Our data in this report showed that O-antigen-negative strains are in addition slightly more sensitive to cationic peptides. It was recently reported that the Helicobacter pylori O antigen is involved in resistance to inorganic pH stress, a condition found in the stomach (39). However, we found no evidence that V. cholerae O-antigen-negative strains (or the core mutants) are more sensitive to inorganic acids (data not shown).

We reported also that galU and galE are involved in the production of the VPS necessary for biofilm formation on abiotic surfaces. Biofilm formation is likely to be important in the life cycle of pathogenic V. cholerae strains, since these strains survive and persist in aquatic ecosystems (8, 16). Commonly, bacteria in aquatic environments are rarely found in the planktonic or free-swimming phase but instead are organized in a three-dimensional biofilm on aquatic surfaces. This may provide an advantage for the bacteria since they are better protected against bactericidal agents, and nutrients may be available (10). Recently, it was reported that V. cholerae O1 El Tor strains develop a three-dimensional biofilm on abiotic surfaces. Furthermore, it was shown that for accelerated attachment to abiotic surfaces, V. cholerae needs a functional flagellum as well as the type IV mannose-sensitive hemagglutinin pilus, and that the development of the three-dimensional biofilm requires production of the VPS (69). The regulation of this complex process is not fully understood.

Constitutive expression of VPS by V. cholerae O1 El Tor leads to a rugose colony phenotype, and these cells are able to form a biofilm on abiotic surfaces (72), probably without the need of the regulatory cascade necessary in wt bacteria. We could enrich for rugose strains by exposure of the motile, biofilm-negative O1 El Tor strain P27459 to the lytic phage K139.cm9. One nonmotile rugose isolate was further characterized, and our results suggested that production of the VPS is responsible for enhanced phage resistance, possibly because the phage cannot penetrate the VPS well enough to bind its receptor, the O antigen. The mutation(s) responsible for constitutive VPS production and the underlying regulatory mechanisms are only poorly understood. However, nonmotility of strain P27459res105 may be the cause of the rugose phenotype, because recent results suggest that the absence of a flagellum leads to constitutive VPS expression, at least in O139 strains (P. Watnick, C. M. Lauriano, K. E. Klose, L. Croal, and R. Kolter, submitted for publication). We discounted an alternate possibility, namely, that the loss of HapR, the activator of HAP, caused the rugose phenotype (28), since we could observe HAP activity in this strain (data not shown).

Our data suggest that UDP-glucose is a precursor in the synthesis of the VPS, since the introduction of a galU mutation into a previously rugose strain led to a nonrugose phenotype, and this strain failed to form a biofilm. Also, the O1 El Tor strain CO970 with a galU mutation was shown to be biofilm negative. The introduction of a mutation in galE into rugose strains also renders them nonrugose, and they are unable to form biofilms on abiotic surfaces, suggesting that VPS synthesis requires UDP-galactose to be synthesized via UDP-glucose in the absence of exogenous galactose (Fig. 6). Thus, we identified in this work two additional enzymes necessary for biosynthesis of the VPS and the formation of V. cholerae biofilms. They are encoded by galU and galE and are required in addition to those encoded by the vps (Vibrio poly saccharide synthesis) region (72).

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