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Identification of a Wzy Polymerase Required for Group IV Capsular Polysaccharide and Lipopolysaccharide Biosynthesis in *Vibrio vulnificus*▽

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The estuarine bacterium *Vibrio vulnificus* is a human and animal pathogen. The expression of capsular polysaccharide (CPS) is essential for virulence. We used a new mini-Tn*10* delivery vector, pNKTXI-SceI, to generate a mutant library and identify genes essential for CPS biosynthesis. Twenty-one acapsular mutants were isolated, and the disrupted gene in one mutant, coding for a polysaccharide polymerase (*wzy*), is described here. A *wecA* gene initiating glycosyltransferase was among the genes identified in the region flanking the *wzy* gene. This, together with the known structure of the CPS, supports a group IV capsule designation for the locus; however, its overall organization mirrored that of group I capsules. This new arrangement may be linked to our finding that the CPS region appears to have been recently acquired by horizontal transfer. Alcian Blue staining and immunoblotting with antisera against the wild-type strain indicated that the *wzy*::Tn*10* mutant failed to produce CPS and was attenuated relative to the wild type in a septicemic mouse model. Interestingly, immunoblotting revealed that the mutant was also defective in lipopolysaccharide (LPS) production. However, the core-plus-one O-antigen pattern typical of *wzy* mutations was apparent. CPS production, LPS production, and virulence were restored following complementation with the wild-type *wzy* gene. Hence, Wzy participates in both CPS and LPS biosynthesis and is required for virulence in strain 27562. To our knowledge, this is the first functional demonstration of a Wzy polysaccharide polymerase in *V. vulnificus* and is the first to show a link between LPS and CPS biosynthesis.

The estuarine bacterium *Vibrio vulnificus* is an opportunistic pathogen of humans and animals (8, 9, 37, 54). Infection occurs via wound contamination or ingestion of raw oysters. The fatality rate of susceptible patients with primary septicemia is greater than 50%, and death often occurs within hours of hospital admission (30, 59, 65). *V. vulnificus* alone is responsible for 95% of all seafood-related deaths in the United States, and it carries the highest death rate of any food-borne disease agent (56).

The expression of capsular polysaccharide (CPS) is essential for virulence in *V. vulnificus* (37). The CPS mediates resistance of bacteria to complement-mediated bacteriolysis and phagocytosis, and translucent, nonencapsulated variants are avirulent (48, 68, 70). Capsule assembly in *Escherichia coli* is the paradigm for capsular assembly in other bacteria (63, 64). Group I and IV capsule biosynthesis parallels lipopolysaccharide (LPS) biosynthesis systems (60). LPS, the major surface molecule of gram-negative bacteria, consists of three distinct structural domains: the O antigen, the core, and lipid A (57, 62). Lipid A linked to the core oligosaccharide is assembled on the inner leaflet of the inner membrane and then translocated to the outer leaflet. The O antigen, which is synthesized via the O-antigen polymerase (Wzy)-dependent pathway, is made up of repeat units containing three to five sugar residues (45). Variations in the composition, sequence, and linkage of the sugars in O antigens give rise to tremendous structural diversity. The O-antigen repeat units are assembled on a lipid carrier, undecaprenol phosphate (und-PP), on the inner side of the inner membrane. Wzx, the O-unit flippase, translocates the individual und-PP repeat units to the periplasm. The und-PP-linked O units are polymerized on the periplasmic face of the inner membrane by the action of Wzy, an integral membrane protein that transfers the nascent polymer from its und-PP carrier to the nonreducing end of the new und-PP-linked O repeat to increase the chain length by one repeat unit. The polymerized O antigen is then ligated to the core oligosaccharide by the integral membrane protein WaaL. The complete LPS is translocated to the external leaflet of the outer membrane. In both group I and group IV CPS biosynthesis, Wzx- and Wzy-dependent systems are used to translocate and synthesize the forming polymer following glycosyltransferase-mediated attachment of monosaccharides to the und-PP carrier. The products of the *wza*, *wzb*, and *wzc* genes are required for the surface expression of group I CPS (23, 24, 46). Wza, Wzb, and Wzc are translocation proteins that export CPS to the outer surface (41, 45). Wza forms a multimeric channel complex in the outer membrane for polymer export (16, 22), Wzc is an inner membrane tyrosine autokinase, and Wzb is its cognate phosphatase (69).

The initiating glycosyltransferase is a distinguishing factor between group I and group IV capsule biosynthesis pathways (63). In group I capsule biosynthesis, WbaP is the initiating transferase that transfers a Gal-1-P or Glc-1-P to und-PP, while WecA catalyzes the initial transfer of GlcNAc-1-P to und-PP in group IV capsule biosynthesis. Carbohydrate com-

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position analysis has shown that *V. vulnificus* possesses multiple capsular types (10), and CPS biosynthesis genes homologous to both group I (12, 66) and group IV (51) CPS operons have been identified in *V. vulnificus*.

The genetic loci for group I and IV CPS production are allelic to many LPS biosynthetic loci, and multiple enzymes may be shared between these pathways. However, little is known regarding the contribution of *V. vulnificus* CPS biosynthesis genes to the synthesis of LPS, primarily due to limitations in detecting *V. vulnificus* LPS by standard silver staining techniques (2, 5, 6). Here, we used transposon mutagenesis and immunodetection to identify genes involved in both CPS and LPS biosynthesis in the clinical isolate ATCC 27562. We cloned and sequenced a 9.3-kb CPS biosynthesis cluster containing eight genes from one acapsular mutant. This region contained a *wzy* polymerase, a *wzx* flippase, a *wecA* initiating glycosyltransferase, and three additional glycosyltransferase genes, supporting a group IV capsule designation for this locus. The marked decreases in the G+C contents of the genes within this region compared to the average G+C content of the organism suggest its recent acquisition by horizontal gene transfer. Agglutination assays and immunoblotting with antisera raised against strain 27562 revealed high-molecular-weight CPS and an O-antigen ladder characteristic of smooth LPS in the wild-type strain. The *wzy*::Tn*10* mutant failed to produce CPS and LPS and was attenuated relative to the wild-type strain in the iron-overloaded septicemic mouse model. Complementation with the wild-type *wzy* gene restored CPS production, LPS production, and virulence to the mutant. Hence, Wzy participates in both CPS and LPS biosynthesis and is required for virulence in strain 27562.

MATERIALS AND METHODS

Bacterial strains and media. *E. coli* S17.1λpir (44) was used as the donor strain for conjugation of the pNKTXI-SceI plasmid into *V. vulnificus*. A rifampin (Rf)-resistant derivative of the encapsulated *V. vulnificus* clinical strain 27562 obtained from the Collection de l'Institut Pasteur was used as the recipient. Both strains were grown on LB media (Sigma). Antibiotics were used at the following concentrations as indicated: ampicillin (Ap), 100 μg/ml; Rf, 100 μg/ml; kanamycin (Km), 25 μg/ml for *E. coli* and 160 μg/ml for *V. vulnificus*. IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 1 mM.

PCR, sequencing, and phylogenetic analysis. PCR was performed with 50-μl volumes, using Platinum Pfx DNA polymerase (Invitrogen) according to the manufacturer's instructions. PCR products were TA cloned into the pCR2.1 vector (Invitrogen) and sequenced at The Centre for Applied Genomics (TCAG) at the Hospital for Sick Children (Toronto, Canada). Alignments were generated with the CLUSTALX (55) software program (version 1.8), and dendrograms were compiled by using the neighbor-joining method (computed from 1,000 independent trials) of CLUSTALW. Phylogenetic trees were generated with TREEVIEW (40).

Construction of pNKTXISce-I. The plasmid pNKBOR (49) contains a mini-Tn*10* transposon (34) but lacks an origin of transfer necessary for conjugation to *V. vulnificus* 27562. Thus, the pNKTXISce-I fusion vector was created through ligation of EcoRI-digested pNKBOR to MfeI-digested pTXISce-IA (Fig. 1). We created the pTXISce-IA vector by introducing an I-SceI site into the multiple cloning site of pTX1A (38).

Conjugations. The pNKTXI-SceI plasmid was transformed into the *E. coli* mobilizing strain S17.1λpir and moved to *V. vulnificus* as follows. Donor and recipient cultures were grown overnight in LB and LB-Rf, respectively. The next day, bacteria were diluted 100 times in fresh media and grown to an optical density at 600 nm (OD_{600}) of 0.7. One milliliter of each was then spun down and washed in LB, followed by incubation at 37°C for 1 h. Cultures were then pelleted, combined, and conjugated overnight without antibiotic selection on LB plates. The following day, cells were resuspended in 5 ml of LB. One milliliter of the resuspension was centrifuged at 10,000 rpm for 2 min, and the pellet was resuspended in 100 μl of LB. Exconjugants with transposon insertions were selected on LB-Rf-Km plates.

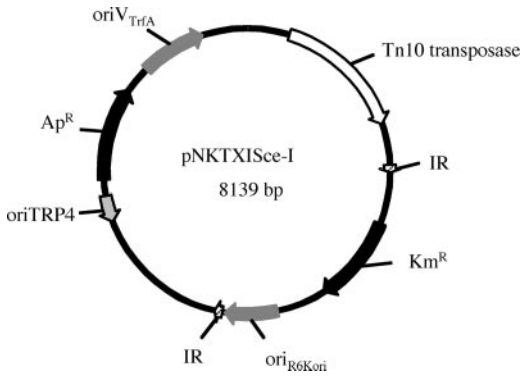

FIG. 1. pNKTXI-SceI mini-Tn10 delivery vector. The pNKTXI-SceI vector was obtained by ligating EcoRI-digested pNKBOR to MfeI-digested pTXISce1A. The vector contains an Ap resistance marker (Ap^r), the Tn*10* transposase, an *oriT*$_{RP4}$ origin of transfer, a *trfA*-dependent origin of replication (*oriV*$_{trfA}$) outside of the minitransposon region, and a π-dependent R6Kγori$_{pir}$ origin of replication within the minitransposon. The minitransposon also carries a Km resistance marker (Km^r). IR, inverted repeats recognized by Tn*10*.

Southern analysis. Southern analysis was performed to verify single insertions of the Tn*10* transposon into the genome of *V. vulnificus*, using the Km resistance marker from the pNKTXI-SceI plasmid as a probe. Genomic DNA was extracted from 21 mutants by using the DNAzol reagent, following the protocol provided by the manufacturer (Invitrogen), and digested with EcoRI. Following electrophoresis on a 1% agarose gel, the DNA was transferred to an N+ nylon membrane (Amersham). The membranes were probed using a Gene Images random primer labeling module (Amersham), following the manufacturer's protocol. Prehybridization, hybridization, and subsequent washes were performed at 55°C. Signal was detected using BioMax MS film (Kodak).

Determination of transposon insertion sites. Translucent *V. vulnificus* colonies were grown overnight on LB-Km plates. Bacteria were scraped from the plate, and genomic DNA was extracted using the DNAzol reagent. The DNA was cut using EcoRI, self-ligated, and transformed into DH5αλpir. Transformants were selected on LB-Km plates. Plasmids were purified using a Sigma GenElute plasmid mini prep kit and sequenced using the primer pirseq (ACACTTAACG GCTGACATGG), designed to obtain sequence going outwards from the R6K origin of replication within the transposon region on the pNKTXI-SceI plasmid. Complete plasmid sequence was obtained by primer walking, and open reading frames (ORFs) were identified using ORFfinder. Putative functions for the disrupted genes were assigned by BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/). Transmembrane helix predictions for the Wzy sequences were obtained using the TMpred online program (http://www.ch.embnet.org/software/TMPRED_form.html).

Construction of pTRC99AoriT$_{RP4}$ expression vector. A NotI site was introduced into the backbone of the expression vector pTRC99A (20) by inverse PCR using the primers pTRC99aNot3 (AAAAGCGGCCGCTTCTCCTTACGCATC TGTGC) and pTRC99aNot4 (AAAAGCGGCCGCAATACCGCATCAGGCG CTC) (underlining indicates the restriction enzyme site). The RP4 origin of transfer was amplified with primers oriT1Not (CTGCGGCCGCCTTTTCCGC TGCATAACCCTG) and oriT2Not (CTGCGGCCGCCGGCCAGCCTCGCA GAGC), using the plasmid pSW23oriT (21) as a template. Both PCR products were cut with NotI, ligated together, and transformed into DH5α to create pTRC99AoriT.

Complementation of the *wzy*::Tn*10* mutant. The sequence of the putative *wzy* gene was amplified from genomic DNA of the 27562 parent strain with primers WzyBamHI (CGGGATCCTCATAAGGGTATGCGTTTGTTA) and WzyFBspHI (GGGTTTTCATGATTACATACAACGTTAGATTAA). The *wzy* gene was cloned into the NcoI and BamHI sites of pTRC99A:oriT. The construct was conjugated to the *V. vulnificus wzy*::Tn*10* mutant as described above, and gene expression was induced with IPTG.

Antiserum production. A 2-liter flask containing 200 ml of LB was inoculated with 1 ml of seed culture suspension (ca. 1×10^9 CFU) of strain 27562, and the bacteria were grown at 30°C for 7 to 8 h with shaking. The bacteria were collected by centrifugation, washed twice with phosphate-buffered saline (PBS; NaCl [130

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mM], Na_2HPO_4 [5 mM], KH_2PO_4 [1.5 mM]), and resuspended in 50 ml of PBS. The number of CFU per ml of suspension (usually ca. 5×10^{10} CFU/ml) was determined by plate counts. The bacteria in 10-ml portions of the suspension were killed by exposure to 0.8% (vol/vol) formalin for 1 day at ca. 25°C and then at 4°C for another 24 h. The bacteria were washed three times in PBS, and the vaccines were stored at 4°C. Complete inactivation was confirmed by testing the growth of suspensions on LB plates at 37°C for 3 days. One-milliliter portions were diluted with sterile PBS to ca. 5×10^9 CFU/ml. Adult New Zealand White rabbits were immunized intravenously with 1 ml of formalin-killed bacteria without adjuvant. Five injections were given on days 1 (0.25 ml), 2 (0.5 ml), 3 (1.0 ml), 4 (1.0 ml), and 11 (1.0 ml). One week after the last immunization, 10 to 20 ml of blood was collected from the marginal ear vein of each rabbit and tested for the presence of antibodies by using the slide agglutination test described below. Following a positive test result, two more boosts of 1.0 ml at 1-week intervals were administered. One week after the second boost, animals were exsanguinated to collect the antisera. The blood was allowed to clot, and the sera were separated and stored at −30°C.

Slide agglutination tests. Overnight cultures of *V. vulnificus* wild-type, *wzy*::Tn*10*, and *wzy*::Tn*10C* cells were diluted to an OD_{600} of 1. Twenty-microliter aliquots of each were applied in duplicate onto microscope slides. One aliquot was mixed with 20 μl of rabbit antisera raised against formalin-killed whole cells of the *V. vulnificus* parent strain. The other aliquot was mixed with sera obtained from the baseline bleed and served as the negative control. Agglutination results were scored after incubation of the mixtures at room temperature for 5 min. A distinct and immediately occurring agglutination was registered as positive, while weak or no agglutination after 5 min was considered a negative test result.

CPS extraction and visualization. The CPS extraction procedure was essentially as described by Gunawardena et al. (29) with some modifications. Single bacterial colonies were inoculated into LB and grown for 18 h at 30°C. Cultures were diluted to an OD_{600} of 0.9, and 1 ml was plated and grown on LB plates for 18 h at 30°C. The cells were collected in 15 ml of LB, pelleted, and resuspended in an equal volume of PBS. Samples were shaken for 1 h at 200 rpm at 25°C. The OD_{600} was measured, and CFU counts were determined after each step to ensure that cell lysis was not occurring and that equal numbers of bacteria were used for CPS isolation. The bacteria were pelleted, and the supernatant was dialyzed against three changes of distilled water (2l for 12 h), followed by concentration through a 10-kDa Amicon Ultra-15 centrifugal filter device column. This was followed by sequential treatment of the retentate with DNase (50 μg/ml plus 1 mM $MgCl_2$), RNase (100 μg/ml), and Pronase E (250 μg/ml) at 2-h intervals at 37°C. The retentate was spun at 100,000 × *g* for 18 h at 20°C and extracted twice with phenol chloroform, and the aqueous layer was dialyzed as described above. The retentate was concentrated using a 100-kDa-molecular-mass-cutoff Centriprep unit (Amicon) spun at 500 × *g* for 1 h. CPS samples were run on a 40% nondenaturing polyacrylamide gel electrophoresis (PAGE) gel or a 4% stacking/12.5% separating Tris-HCl-glycine sodium dodecyl sulfate (SDS)-PAGE gel, using the procedure described by Laemmli (36). CPS was visualized by immunostaining using antisera against formalin-killed whole cells of *V. vulnificus* 27562 and Alcian Blue staining of acidic polysaccharides as described by Pelkonen et al. (42, 43).

LPS extraction and visualization. Whole-cell lysates were prepared by following the method of Hitchcock and Brown (32) with several modifications. Five-milliliter cultures were grown overnight in appropriate antibiotics, and the OD_{600} was adjusted to 0.9. Five milliliters of each was centrifuged at 5,000 rpm for 10 min, and the cells were washed six times in PBS. The pellet was resuspended in 375 μl of HB buffer (1% SDS, 4% β-mercaptoethanol, 10% glycerol, 1 M Tris-HCl, pH 6.8, 0.002% bromophenol blue) and boiled for 30 min. Proteinase K (1.5 μl; 20 mg/ml) was then added, and the reaction mixtures were incubated at 56°C overnight. The LPS samples were boiled for 15 min and run on a 4% stacking/12.5% separating Tris-glycine SDS-PAGE gel. LPS was visualized by immunoblotting.

LD_{50} determinations. Strains were inoculated from single colonies into LB and were cultured overnight at 30°C. The overnight culture was seeded at a 1:100 dilution into fresh media and incubated at 30°C until the bacteria reached an OD_{600} of 1.0. Bacteria were collected by centrifugation and washed once with PBS. Bacterial-cell concentrations were confirmed by plate counts. Five-week-old female CD-1 mice housed under pathogen-free conditions were pretreated with an intraperitoneal injection of iron dextran (250 μg of iron-dextran per g of body weight) 60 min before inoculation with the bacterial strains. Groups of mice ($n = 5$) were injected intraperitoneally with 0.1 ml of serial log dilutions (10^2 to 10^8 CFU) of each bacterial strain. Injection of PBS alone served as a control. Mice were observed for up to 24 h postinfection with the recording of deaths or moribund animals. The 50% lethal dose (LD_{50}) was quantified using the simplified linear approximation method of Reed and Muench (47).

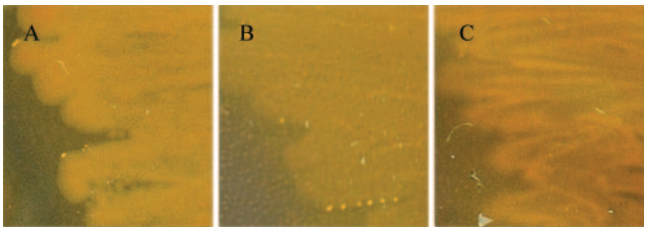

FIG. 2. Restoration of CPS production in the *wzy*::Tn*10* mutant by the wild-type *wzy* gene. (A) Wild-type strain 27562; (B) *wzy*::Tn*10* mutant; (C) *wzy*::Tn*10* mutant complemented with the wild-type *wzy* gene.

Nucleotide sequence accession number. The sequence determined for this study has been deposited in GenBank under accession number EU179506.

RESULTS

Transposon mutagenesis with the pNKTXISce-I mini-Tn10 delivery plasmid. To facilitate the generation of transposon mutants and the identification of the disrupted genes, we created the mini-Tn*10* delivery plasmid pNKTXISce-I (Fig. 1). This vector was generated by fusing the plasmid backbones of pNKBOR and pTXISce-IA. pNKTXISce-I carries a Km^r marker and a π-dependent origin of replication in its mini-Tn*10* transposon, while a TrfA-dependent origin of replication is present in the vector backbone. Thus, this plasmid can be maintained and its copy number controlled in cells expressing either the π (35, 39) or TrfA (26, 27) protein. The presence of an origin of replication in the mini-Tn*10* transposon also allows for the isolation of the transposon insertion site and flanking DNA as Km-resistant clones in *E. coli* strains expressing the π protein. It also carries an RP4 origin of conjugative transfer for conjugation of the plasmid to a wide range of bacteria and an I-SceI restriction enzyme site for plasmid verification and cloning of additional modules. The plasmid is fully defined, as the complete sequences of both parent plasmids are known, and the Tn*10* insertions are stable.

Identification of a capsule biosynthesis locus in *V. vulnificus* 27562. Transposon mutagenesis was conducted as described in Materials and Methods. Acapsular mutants were selected from the conjugation experiments, and Southern analysis was used to verify that each mutant contained a single transposon insertion in its chromosome. The transposon insertion points were identified by sequencing and BLAST analysis. The disrupted gene in one mutant (Fig. 2) was identified as a putative polysaccharide polymerase (*wzy*). Subsequently, 9.3 kb of the region surrounding the insertion site was cloned, sequenced, and annotated (Fig. 3). Potential functions were assigned based on homology to previously described genes (Table 1). The ORFs appeared to encode gene products common to CPS/O-antigen biosynthesis clusters, including four glycosyltransferases, a putative *wzy* polymerase, and a *wzx* flippase homologue. Of the eight ORFs identified, seven appeared to be transcribed in the same orientation. This organization is reminiscent of the operon-like structure common to CPS biosynthesis clusters in a variety of encapsulated bacterial species. The lone exception encoded a homologue of WecA, the initiating glycosyltransferase that in part defines group IV CPS biosynthesis loci (60).

The complete Wzy protein was analyzed using TMpred soft-

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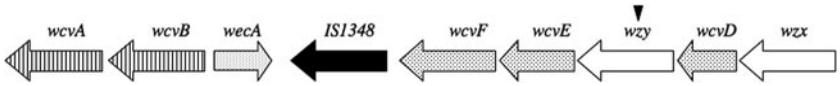

FIG. 3. *V. vulnificus* 27562 capsule polysaccharide biosynthesis cluster. Open arrows represent the locations and directions of transcription of the respective ORFs. Horizontally striped, spotted, black, and white arrows indicate nucleotide sugar biosynthesis, transferase, insertion sequence, and processing genes, respectively. The closed triangle denotes the transposon insertion site. Gene characteristics are summarized in Table 1.

ware. The result was compared with those of Wzy proteins from *Escherichia coli* (GenBank accession no. AAO37717.1), *Yersinia enterocolitica* (GenBank accession no. AAC60768), *Shigella boydii* (GenBank accession no. AAW29815), *Salmonella enterica* (GenBank accession no. CAA43077), and *Bacillus cereus* (GenBank accession no. AAS44289.1). Eleven inside-to-outside transmembrane helices were suggested for the putative *V. vulnificus* Wzy protein. This was in agreement with the 10 or 11 transmembrane helices predicted for the Wzy proteins of *E. coli*, *Y. enterocolitica*, *S. boydii*, *Salmonella enterica*, and *B. cereus*. Phylogenetic comparisons with previously described bacterial Wzy and WaaL enzymes (Fig. 4) also supported the designation of a Wzy polymerase function for the disrupted gene.

Demonstration of a *wzy*-dependent CPS biosynthesis pathway in *V. vulnificus*. To verify that disruption of the *wzy* gene prevented CPS production, we conducted agglutination assays with the wild-type and mutant strains. Antisera against formalin-killed whole cells reacted strongly with the 27562 parent strain in slide agglutination tests, while no agglutination was observed with the *wzy*::Tn*10* mutant. A positive agglutination reaction was obtained when the pTRC99aoriT expression vector carrying the wild-type *wzy* gene was introduced into the *wzy*::Tn*10* mutant, while no agglutination was detected when the *wzy*::Tn*10* mutant carried the empty pTRC99AoriT vector. CPS was extracted from the wild type, the *wzy*::Tn*10* mutant, and the *wzy*::Tn*10* mutant strain that was complemented with the wild-type *wzy* gene (*wzy*::Tn*10*C). The CPS was then separated by PAGE and visualized by Alcian Blue staining for acidic polysaccharides and immunoblotting (Fig. 5 and 6). Both detection methods revealed the presence of a high-molecular-weight polysaccharide and a prominent lower-molecular-weight product (designated the K band) in the wild-type strain, while no such material was observed in the *wzy*::Tn*10* mutant. Complementation of the *wzy*::Tn*10* mutant with the wild-type *wzy* gene restored production of the high-molecular-weight polysaccharide and the K band. These results confirmed the absence of CPS inferred from the translucent phenotype of the mutant and supported the involvement of the *wzy* gene in CPS production in *V. vulnificus* 27562.

Wzy is involved in LPS biosynthesis. To investigate whether the *wzy* CPS mutant was also defective in LPS production, LPS was extracted from the wild-type, the *wzy*::Tn*10* mutant, and the *wzy*::Tn*10*C strains and separated by SDS-PAGE. Similar to previous reports (2, 5, 6), silver staining failed to detect the characteristic ladder pattern observed for smooth LPS profiles among the *Enterobacteriaceae* (data not shown). However, immunoblotting using antisera against the wild-type strain revealed a ladder pattern characteristic of smooth LPS. Two fast-migrating bands corresponding to the lipid A-core and the core-plus-one O-antigen structures were also observed (Fig. 7). Attempts to better resolve the fastest-migrating bands by using the Novex Tricine and 4 to 20% Bis-Tris gel systems were unsuccessful. The same prominent K band present in the CPS sample from wild-type bacteria was also evident in the LPS from this strain. Neither the ladder nor the K band was present in the LPS extract of the *wzy*::Tn*10* mutant. Complementation with the wild-type *wzy* gene resulted in the reappearance of both the ladder and the K band in the LPS gels. An increase in the intensity of the banding pattern was observed following IPTG induction. In particular, the accumulation of higher-molecular-weight bands in the LPS ladder following IPTG induction supports a polymerization activity for the *wzy* gene. The empty vector did not result in the reappearance of the banding pattern. The results indicated that Wzy was involved in the production of both CPS and the O-antigen in *V. vulnificus*.

The *wzy*::Tn*10* mutant is attenuated in vivo. The LD_{50} of *V. vulnificus* is dramatically reduced in the presence of iron, and the iron-overloaded mouse model is widely used to demon-

TABLE 1. Putative functions of the *V. vulnificus* CPS proteins

Gene or insertion element	% G+C	Putative function	Organism	E value	GenBank accession no.
wcvA	42.0	Nucleotide sugar epimerase	*Vibrio vulnificus* 1003 (O)	0	AF499932.1
wcvB	41.7	Nucleotide sugar dehydrogenase	*Vibrio vulnificus* 1003 (O)	0	AAO32664.1
wecA	41.4	Undecaprenylphosphate *N*-acetylglucosamine 1-phosphate transferase	*Vibrio vulnificus* 1003 (O)	5e−130	AAO32663.1
IS*1358*	43.0	Putative transposase	*Vibrio cholerae*	6e−147	AAC82490.1
wcvF	38.3	Rhamnosyltransferase	*Shewanella frigidimarina* NCIMB 400	3e−132	ZP_00638805
wcvE	32.5	Glycosyltransferase	*Methanosarcina mazei* Go1	3e−8	CAI34411
wzy	31.2	CPS polymerase	*Bacillus cereus* ATCC 10987	3e−21	NP_981681
wcvD	32.8	Putative glycosyltransferase	*Streptococcus pneumoniae*	5e−33	CAI34411
wzx	30.8	Polysaccharide translocase	*Bacillus thuringiensis* serovar *israelensis* ATCC 35646	1e−23	NZ_AAOX01000001

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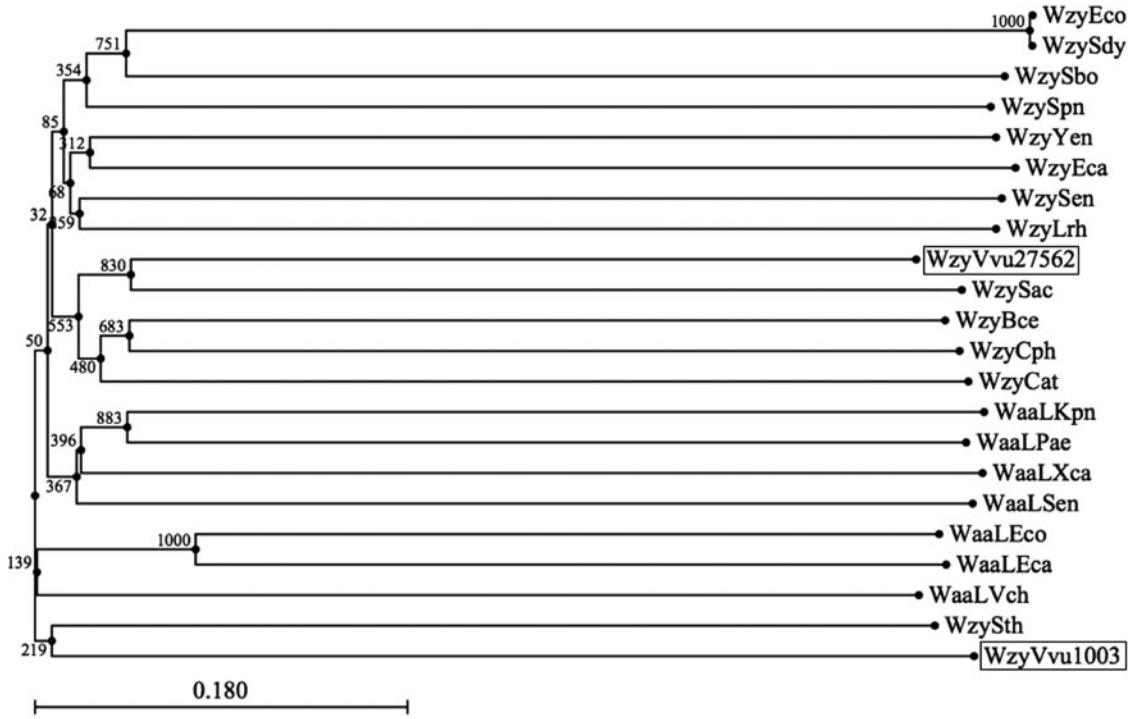

FIG. 4. Phylogenetic relationship of bacterial Wzy and WaaL enzymes. An unrooted dendrogram based on representative Wzy and WaaL bacterial enzymes is shown. The organism abbreviations and GenBank accession numbers (in brackets) are as follows: Bce, *Bacillus cereus* (NP_981681); Sac, *Syntrophus aciditrophicus* (YP_460441); Sth, *Streptococcus thermophilus* LMG 18311 (YP_139549); Spn, *Streptococcus pneumoniae* (AAK20717); Cph, *Clostridium phytofermentans* ISDg (ZP_01352529); Cat, *Croceibacter atlanticus* HTCC2559 (ZP_00951257); Eca, *Erwinia carotovora* subsp. *atroseptica* SCRI1043 (YP_048289); Eco, *Escherichia coli* (AAO39700); Sbo, *Shigella boydii* (AAV41072); Sdy, *Shigella dysenteriae* (AAR97964); Lrh, *Lactobacillus rhamnosus* (AAW22494); Sen, *Salmonella enterica* (CAA43077); Pae, *Pseudomonas aeruginosa* PAO1 (AAG08384); Vch, *Vibrio cholerae* (AAL77359); Vvu, *V. vulnificus*; Kpn, *Klebsiella pneumoniae* (AAD37765); Xca, *Xanthomonas campestris* pv. *vesicatoria* strain 85-10 (YP_365325); Yen, *Yersinia enterolitica* (AAC60768). The two *V. vulnificus* Wzy polymerases are boxed, and the bootstrap values for each node are indicated.

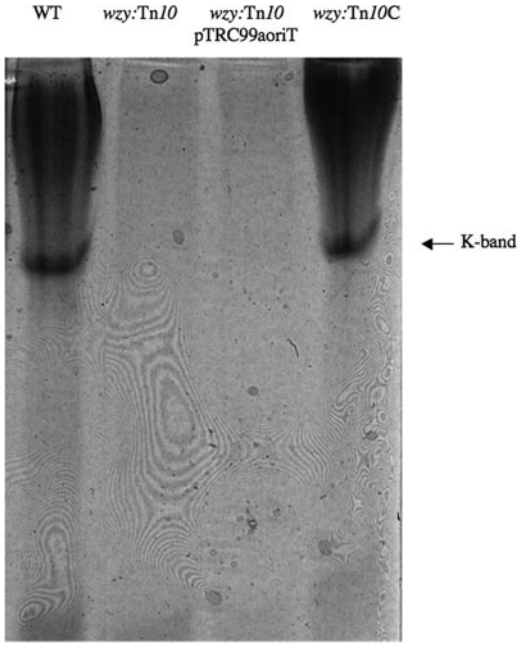

FIG. 5. Alcian Blue stain of *V. vulnificus* CPS. CPS was extracted from wild-type (WT), *wzy*::Tn*10* mutant (*wzy* polymerase knockout), and *wzy*::Tn*10*C (*wzy*::Tn*10* complemented with pTRC99aoriT::*wzy*) cells and separated on a nondenaturing polyacrylamide gel. CPS was visualized by Alcian Blue staining.

strate the enhanced in vivo virulence of *V. vulnificus* strains in the presence of excess serum iron (67). The LD_{50} values for the wild type and the *wzy*::Tn*10* and *wzy*::Tn*10*C mutant strains were determined. The LD_{50} of the wild-type strain (ATCC 27562) was 2.4×10^4. Disruption of the *wzy* gene (*wzy*::Tn*10*) caused an increase in the LD_{50} to $>4.2 \times 10^7$. Virulence was restored to the attenuated mutant following complementation with the wild-type gene (*wzy*::Tn*10*C; LD_{50}, 1.4×10^4). Thus, the activity of the Wzy polymerase is required for virulence in *V. vulnificus*.

DISCUSSION

CPS expression is essential for *V. vulnificus* virulence (68, 70). There are several studies identifying genes involved in the biosynthesis of CPS in *V. vulnificus* strains (12, 51, 66, 71); however, most of these studies provide only similarity-based assignments for the identified genes. This study endeavored to provide functional support for proteins involved in CPS biosynthesis in *V. vulnificus*. Acapsular transposon mutants were analyzed, and the disrupted gene in one of these mutants was shown to code for a polysaccharide polymerase (Wzy) based on the following observations: (i) although Wzy polymerases display limited sequence similarity and they share membrane topology features and motifs with WaaL ligases (45, 50), the

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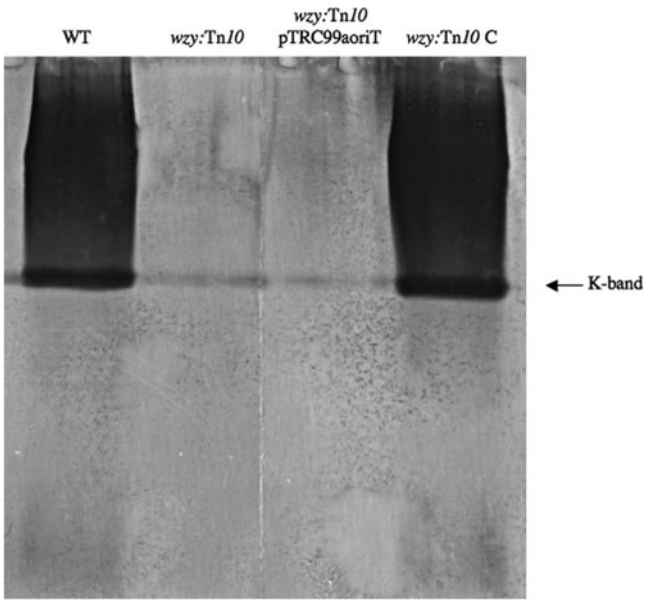

FIG. 6. Immunoblot of *V. vulnificus* CPS. CPS was extracted from wild type (WT), *wzy*::Tn*10* mutant (*wzy* polymerase knockout), and *wzy*::Tn*10*C (*wzy*::Tn*10* complemented with pTRC99aoriT:*wzy*) cells and separated by denaturing SDS-PAGE. CPS was visualized using immunoblotting with antisera to WT formalin-killed whole cells.

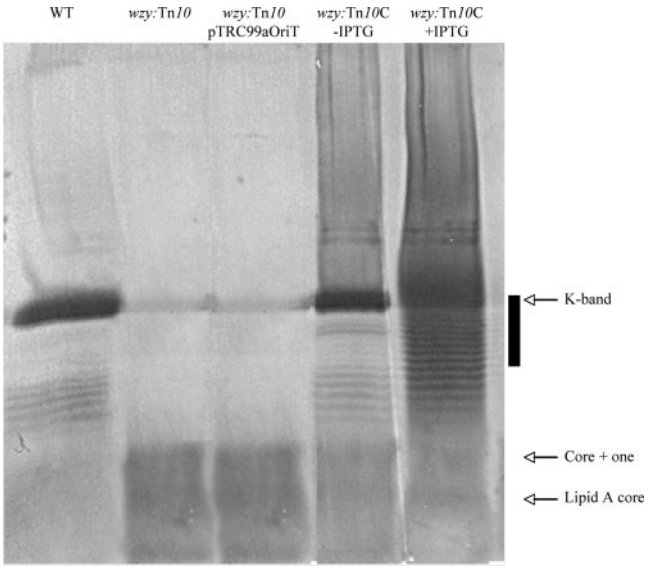

FIG. 7. Immunoblot of *V. vulnificus* LPS. LPS was extracted from wild-type (WT), *wzy*::Tn*10* mutant (*wzy* polymerase knockout), and *wzy*::Tn*10*C (*wzy*::Tn*10* complemented with pTRC99aoriT::*wzy*) cells. The LPS was separated on a denaturing SDS-PAGE gel and visualized by immunoblotting. Expression of the *wzy* gene was induced by the addition of IPTG from the media (+IPTG). The position of the K band band is shown. The black bar denotes the higher-molecular-weight O-antigen bands that accumulate upon induction of *wzy*.

core-plus-one O-antigen pattern typical of *wzy* mutations (1, 31) was apparent in our LPS immunoblots; (ii) the Wzy enzyme was partitioned within a phylogenetic clade composed of other Wzy enzymes; (iii) if it is assumed that polysaccharide polymerases and ligases lack substrate specificity (28), then it follows that increases in polymerase activity result in the accumulation of longer polysaccharide polymers being attached to the lipid A core, while increases in ligase activity result in the accumulation of shorter polysaccharide polymers being attached to the lipid A core. The increased O-antigen banding pattern, and in particular the accumulation of higher-molecular-weight bands in the LPS profile of the *wzy*::Tn*10*C strain, is consistent with a polymerase function for Wzy. The attenuated phenotype of the *wzy*::Tn*10* mutant in the iron-overloaded septicemic mouse model demonstrated that Wzy was required for virulence. Immunoblotting revealed that the *wzy*::Tn*10* mutant was also defective in LPS production. Complementation with the wild-type gene restored CPS production, LPS production, and virulence to the mutant. Hence, Wzy is a polysaccharide polymerase that participates in the production of both CPS and LPS in *V. vulnificus* 27562. To our knowledge, this is the first study to functionally demonstrate the activity of a Wzy CPS polymerase in *V. vulnificus*.

A new genetic organization for the group IV CPS locus of *V. vulnificus* 27562. Both group I and group IV CPS biosynthesis loci code for Wzy-dependent systems that include a Wzx flippase; however, several differences have been used to discriminate these CPS groups. In group I CPS biosynthesis, WbaP is the initiating transferase and the *wza*, *wzb*, *wzc*, *wzx*, and *wzy* genes reside within the same genetic locus. This feature distinguishes group I Wzy-dependent loci from O-antigen loci (60). In group IV CPS biosynthesis, the initiating transfer is catalyzed by WecA and the *wza*, *wzb*, and *wzc* genes are found at a chromosomal location different from that of the *wzx* and *wzy* genes, making group IV CPS loci allelic to many Wzy-dependent O-antigen loci (60). Despite this, the *wza*, *wzb*, and *wzc* genes still participate in group IV capsule assembly, as recently demonstrated with *E. coli* (41).

Based on the location of the *wza*, *wzb*, and *wzc* genes, a group I capsule was proposed for several *V. vulnificus* strains (12, 66). BLAST analysis indicated that neither *V. vulnificus* strain CMCP6 nor YJ016 contained a *wecA* homologue, supporting their group I classification (12, 66). Homologues of *wecA*, *wzy*, and *wzx* were identified in a CPS cluster in *V. vulnificus* 1003 (O) (51). Based on the presence of *wecA*, a group IV capsule was proposed for this strain; however, the chromosomal location of these genes relative to *wza*, *wzb*, and *wzc* was not determined. Furthermore, functional assignments for the ORFs were similarity based; none were shown to catalyze the proposed reaction, and none of the disrupted genes were genetically complemented with their functional counterparts to demonstrate their specific role in CPS production. Hence, a CPS grouping for the capsule could not be assigned with certainty. The CPS of strain 27562 is a serine-linked polysaccharide composed of D-GlcNAc, MurNAc, D-GalA, and L-Rha (29). The 9.3-kb CPS loci identified here contained *wecA*, *wzx*, and *wzy* genes, and we have demonstrated that Wzy is a functional polysaccharide polymerase. Southern analysis and sequencing have indicated that the *wza*, *wzb*, and *wzc* genes reside within the same genetic locus as the *wecA*, *wzx*, and *wzy* genes that we identified in strain 27562 (A. Nakhamchik, C. Wilde, and D. A. Rowe-Magnus, unpublished results). Hence, this strain appears to have a group IV Wzy-dependent CPS with a locus organization that mirrors that of group I CPS loci.

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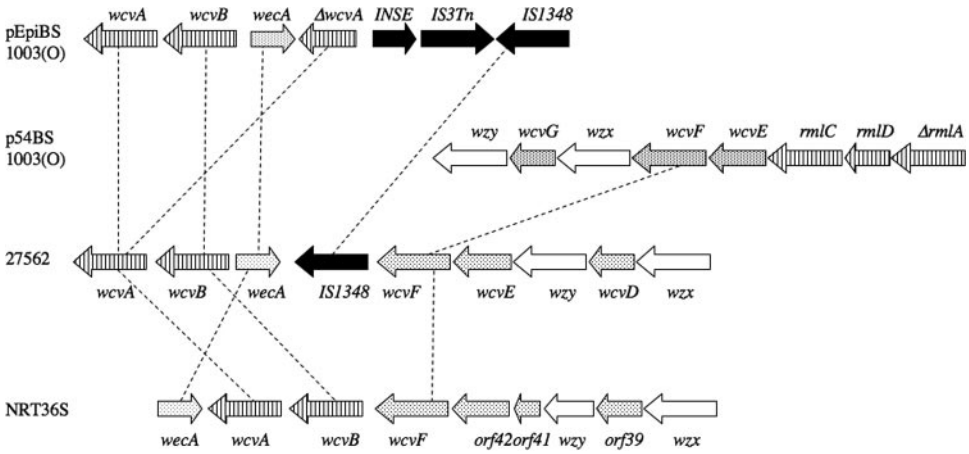

FIG. 8. Genomic comparison of the group IV capsule loci of *V. vulnificus* strains 27562 and 1003 (O) and *V. cholerae* NRT36S. Open arrows represent the locations and directions of transcription of the respective ORFs. Horizontally striped, spotted, black, and white arrows indicate nucleotide sugar biosynthesis, transferase, insertion sequence, and processing genes, respectively. Dotted lines highlight the similar gene arrangements of the strains. The gene designations for strains 1003 (O) and NRT36S are as described in references 51 and 14.

A comparison of the CPS biosynthesis regions of strains 27562 and 1003 (O) revealed that the two loci were similar but not identical in organization (Fig. 8). In 1003 (O), the *wcvF* rhamnosyltransferase is 5′ of *wzx*, while it is 3′ of *wzx* in 27562. IS*1348*, *wecA*, *wcvB*, and *wcvA* occur in the same order in the two strains, but strain 1003 (O) also contains *INSE*, IS*3Tn*, and a nonfunctional *wcvA* duplication. The presence of *wecA* and a rhamnosyltransferase gene, *wcvF*, in strain 27562 accounts for two of the transfer activities required for the assembly of its CPS. Transfer of the D-GalA and MurNAc moieties could be catalyzed by the *wcvD* and *wcvE* transferases to complete the structure.

Recent acquisition of the CPS biosynthesis cluster. The new arrangement that we observed for the group IV CPS locus of strain 27562 may be linked to our finding that the CPS region appears to have been recently acquired by horizontal transfer. While the average G+C content for *V. vulnificus* strains CMCP6 and YJ016 is 46.7% (13), the G+C contents of the genes in the CPS cluster identified here dropped from 41.7% for the *wcvB* gene to 30.8% for the *wzx* gene (Fig. 2). The five genes with the lowest G+C contents (*wzx*, *wcvD*, *wzy*, *wcvE*, and *wcvF*) were adjacent to IS*1358*, appear to be organized as an operon, and showed the highest similarity to genes from gram-positive or archaeal organisms (Table 1). The horizontal acquisition of the CPS biosynthesis clusters can clearly contribute to the tremendous structural diversity observed for the CPS of *V. vulnificus* clinical and environmental isolates and may play an important role in the evolution of new strains, similar to what has been observed for *Vibrio cholerae* O139. The epidemic *Vibrio cholerae* O139 serovar emerged from the pandemic biotype El Tor O1 serovar through the replacement of a 22-kb region by a 40-kb O139-specific DNA fragment (7) that contained the IS*1358* insertion sequence. IS*1358* was shown to be active for transposition (25) and is also associated with LPS loci in serovars of *V. cholerae* (52, 53), *V. vulnificus* (51), and *Vibrio anguillarum* (33, 53), making it possible to envision IS*1358*-mediated genetic exchange between *Vibrio* species that leads to the genesis of new capsule regions. It is tempting to speculate that IS*1358* participated in the acquisition of the CPS loci in strain 27562; however, low-G+C-content polysaccharide biosynthesis genes are found on both sides of IS*1358* and only a single copy of IS*1358* is present on the chromosome (A. Nakhamchik and D. Rowe-Magnus, unpublished results). At present, the role, if any, of IS*1358* in the evolution of CPS clusters in the *Vibrionaceae* remains unclear.

Does *V. vulnificus* harbor a hybrid CPS/LPS biosynthetic cluster? Wzy polymerases participate in O-antigen and CPS (K-antigen) production in group I and group IV capsules (3, 4, 23, 61, 63). Hence, it is possible for the same Wzy polymerase to be involved in the production of both the O and K antigens in the same strain. *E. coli* O9a:K30 has the same antigen, K30, present in two forms: as a smaller polymer attached to lipid A (K_{LPS}) and as a capsular antigen. A Wzy polymerase mutation in this strain results in the loss of both the $K30_{LPS}$ and the capsular K30 antigen (23). Campbell et al. isolated *Sinorhizobium meliloti* mutants that were defective in the production of K antigen; these mutants also had an altered LPS profile (11). The LPS and CPS of *V. cholerae* O139 are structurally identical (19, 58), and the functions for the biosynthesis of the O antigen and the CPS were shown to be dependent upon shared genes that were carried on the same locus (17, 18). Interestingly, the overall organization (Fig. 8) and effect of transposon insertions on CPS and LPS biosynthesis that we observed in strain 27562 paralleled those reported for *V. cholerae* NRT36S (14). In NRT36S, the CPS and LPS biosynthesis genes share the same genetic locus. It was proposed that such an arrangement could play a key role in the evolution of new *V. cholerae* strains by providing a mechanism for the simultaneous emergence of new K and O antigens in a single strain. This notion is supported by the isolation and characterization of genetically similar strains of *V. parahaemolyticus* that express entirely different O and K antigens (15). Our results clearly demonstrate a link between CPS and LPS production in *V. vulnificus* 27562. Thus, it is conceivable that enzymes common to these two biosynthetic pathways are encoded by the same locus in this strain. We are examining the effects of targeted, nonpolar knockouts of other genes in this region on CPS and O-antigen production, and we are determining the structure of its O antigen.

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Does *V. vulnificus* produce a K_{LPS}? The persistence of the K band in both the LPS and CPS preparations was unexpected. The K band remained in the LPS preparations despite the performance of six PBS washes. Hence, it is tightly linked to the cell surface rather than loosely associated with it. This suggested that either the K band represents an O antigen of a preferred chain length whose size is governed by an as yet unidentified chain length determinant (23, 69) or strain 27562 produces an additional polysaccharide (K_{LPS}) that is linked to lipid A in a manner similar to that for the K_{LPS} in *E. coli* serotype K40 (3, 60). The presence of the K band in CPS preparations can be best explained by the production of a K_{LPS}. The OD_{600} and CFU counts were monitored before and after the PBS wash during preparation of the CPS. Both the OD_{600} and CFU/ml values increased, indicating that the bacteria remained intact and viable. Thus, the release of LPS due to cell lysis was not the source of the K band in the CPS preparation. Furthermore, a 100-kDa-cutoff Amicon Centriprep column was used to minimize LPS cross-contamination of the CPS. We believe that the K band produced by *V. vulnificus* 27562 is actually a K_{LPS} and its presence in the CPS preparations is a result of unlinked K_{LPS} associating with the higher-molecular-weight CPS during purification.

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