

Genetic Variation in the *Vibrio vulnificus* Group 1 Capsular Polysaccharide Operon

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***Vibrio vulnificus* produces human disease associated with raw-oyster consumption or wound infections, but fatalities are limited to persons with chronic underlying illness. Capsular polysaccharide (CPS) is required for virulence, and CPS expression correlates with opaque (Op) colonies that show “phase variation” to avirulent translucent (Tr) phenotypes with reduced CPS. The results discussed here confirmed homology of a *V. vulnificus* CPS locus to the group 1 CPS operon in *Escherichia coli*. However, two distinct *V. vulnificus* genotypes or alleles were associated with the operon, and they diverged at sequences encoding hypothetical proteins and also at unique, intergenic repetitive DNA elements. Phase variation was examined under conditions that promoted high-frequency transition of Op to Tr forms. Recovery of Tr isolates in these experiments showed multiple genotypes, which were designated TR1, TR2, and TR3: CPS operons of TR1 isolates were identical to the Op parent, and cells remained phase variable but expressed reduced CPS. TR2 and TR3 showed deletion mutations in one (*wzb*) or multiple genes, respectively, and deletion mutants were acapsular and locked in the Tr phase. Complementation in *trans* restored the Op phenotype in strains with the *wzb* deletion mutation. Allelic variation in repetitive elements determined the locations, rates, and extents of deletion mutations. Thus, different mechanisms are responsible for reversible phase variation in CPS expression versus genetic deletions in the CPS operon of *V. vulnificus*. Repetitive-element-mediated deletion mutations were highly conserved within the species and are likely to promote survival in estuarine environments.**

Vibrio vulnificus causes systemic human infections with high mortality (>50%), and death can occur within hours or days following exposure (3). Illness is generally a consequence of raw oyster consumption or exposure of wounds to seawater. Underlying liver disease, diabetes, hemochromatosis (iron overload), and immune system dysfunction are prerequisites for life-threatening infections. The pathogenesis of *V. vulnificus* has been reviewed (14, 54), and disease symptoms resemble endotoxic shock. Although the contribution of lipopolysaccharide (LPS) to virulence remains unclear (31, 38), lethality in animal models is clearly related to capsular polysaccharide (CPS) expression (38, 49, 62, 63, 64, 69). Both CPS expression and virulence are associated with opaque (Op) colony morphology, but colonies can spontaneously revert to the translucent (Tr) phenotype in a process termed phase variation. Tr colonies have reduced CPS expression and diminished virulence in mouse models (64). These phenotypes are reversible for both Op→Tr and Tr→Op transitions at rates of about 10^{-3} to 10^{-4} , and colony morphology is currently the most reliable virulence marker for *V. vulnificus* (51).

Bacterial phase variation typically is related to reversible mutations that alter the expression of cell surface structures (CPS, LPS, pili, flagella, or outer membrane proteins) and increase resistance to host defenses or enhance survival under stressful environmental conditions (for reviews, see references 17 and 57). Phase-variable genetic events serve as an on/off switch for expression of a given phenotype. Distinct mechanisms for phase variation have been described and include

inversions (13, 29), differential DNA methylation (42), and recombination mediated by insertion elements (36) or by repetitive DNA structures (52, 56). Repetitive-DNA-mediated phase variation involves tandem repeats located at intragenic or promoter-associated sites and results from strand slippage and mismatch during replication and DNA repair (32, 53). Insertions or deletions within intragenic repetitive elements lead to reversible frameshift mutations, while alterations within the promoter alter promoter strength (46).

A CPS operon was identified in *V. vulnificus* that showed homology to CPS operons previously categorized in *E. coli* (62). According to Whitfield and Roberts (61), CPS operons are classified into groups 1 through 4 based on CPS biochemistry and genetic organization. The *V. vulnificus* CPS operon shows the closest homology to group 1 due to the presence of *wza-wzb-wzc* genes, which are highly conserved among the *Enterobacteriaceae* (39, 60, 61). Diverse CPS composition was described for *V. vulnificus* (5), but most encapsulated strains (88%) were positive for group 1 by DNA hybridization (62). Specific functions of the proteins encoded by the conserved *wza-wzb-wzc* genes have been determined in *E. coli*. Wza is an outer membrane lipoprotein that multimerizes to form ringlike structures for secretion of high molecular weight CPS polymers across the outer membrane (11, 12). Mutations in the *wza* gene led to loss of polymer secretion and accumulation of high-molecular-weight intermediates within the periplasm in both *E. coli* (12) and *V. vulnificus* (62). Following *wza* are two genes whose encoded proteins are thought to have a synergistic function: Wzc is a tyrosine autokinase, and Wzb is the cognate phosphatase (11, 66). In *E. coli* K30, these proteins are required for the assembly of the high-molecular-weight capsular layer on the cell surface (12).

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TABLE 1. Summary of *V. vulnificus* strains

Strain	CPS allele ^a	Description ^b	Reference
Clinical			
MO6-24	1	Encapsulated, virulent (blood)	62
LC4	1	Encapsulated, virulent (blood)	64
C7184	1	Encapsulated, virulent (blood)	65
E4125	1	Encapsulated, virulent (blood)	65
CMCP6	1	Encapsulated, virulent (blood)	NC_004459
CDC9003-97	1	Encapsulated, virulent (blood)	34
CDC9038-96	1	Encapsulated, virulent (blood)	34
CDC9074-96	1	Encapsulated, virulent (blood)	34
CDC9053-96	1	Encapsulated, virulent (blood)	34
CDC9067-96	1	Encapsulated, virulent (blood)	34
ATL9824	1	Encapsulated, virulent (blood)	34
CDC9070-06	1	Encapsulated, virulent (blood)	34
CDC9352-94	1	Encapsulated, virulent (blood)	34
CDC9342-95	2	Encapsulated, virulent (blood)	34
YJ016	2	Encapsulated, virulent (hospital)	7
Environmental			
99-743DP-B6	1	Encapsulated, virulent (oyster)	34
99-509DP-A6	1	Encapsulated, virulent (oyster)	34
99-505DP-C8	2	Encapsulated, virulent (oyster)	34
99-742DP-A9	2	Encapsulated, virulent (oyster)	34
99-623DP-F5	2	Encapsulated, virulent (oyster)	34
99-537DP-G7	2	Encapsulated, virulent (oyster)	34
99-581DP-C7	2	Encapsulated, virulent (oyster)	34
99-520DP-B8	2	Encapsulated, virulent (oyster)	34
99-622DP-E4	2	Encapsulated, virulent (oyster)	34
99-736DP-C7	2	Encapsulated, virulent (oyster)	34
99-624DP-C10	2	Encapsulated, virulent (oyster)	34
99-540DP-B6	2	Encapsulated, virulent (oyster)	34
99-505DP-C8	2	Encapsulated, virulent (oyster)	34
99-796DP-E7	2	Encapsulated, virulent (oyster)	34
99-625DP-D8	2	Encapsulated, virulent (oyster)	34

^a Alleles 1 and 2 refer to alternate DNA sequences in group 1 CPS operons as described in text.

^b Strain description includes CPS expression, virulence, and source of isolation from clinical septicemia (blood) or environmental origin (oyster).

The genetic basis for phase variation in *V. vulnificus* is unknown. We hypothesized that rearrangements within the group 1 CPS operon may be responsible for Op/Tr transitions in CPS phenotypes, as DNA polymorphisms were observed previously at this locus among phase variants of *V. vulnificus* (62). The present study monitored genetic mutation at this locus in relation to phase variation. Genetic diversity of CPS genes among strains from clinical and environmental origin was also examined. Culture conditions similar to those previously reported to induce high-frequency phase variation for *Vibrio cholerae* (1) facilitated the recovery of altered colony types, and the results indicated that multiple genetic alterations are responsible for transitions in the expression of the group 1 CPS operon.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *V. vulnificus* strains (Table 1) were grown in Luria-Bertani broth (LB) at 37°C or on LB agar (LA) at 30°C and stored at -70°C in LB with 50% glycerol. When required, kanamycin (50 µg/ml), tetracycline (10 µg/ml), and polymyxin (50 µg/ml) were added to cultures of *V. vulnificus*. *Escherichia coli* S17-1 λpir (48) and JM109 (Promega) were used for complementation and cloning experiments and were grown as described above but with tetracycline (30 µg/ml) and ampicillin (100 µg/ml). Unless otherwise stated, media were purchased from Difco and chemicals from Sigma.

Induction of phase variation. To investigate transitions in colony types for Op or Tr isolates of *V. vulnificus*, individual colonies were inoculated in LB and grown overnight at 37°C with shaking. Standardized inocula (10⁶ CFU/ml) were derived from serial dilutions in phosphate-buffered saline (PBS) of washed cells (three times in PBS) from the overnight cultures and transferred into media (45

ml) in 250-ml flasks prepared with either 1% proteose peptone no. 3 at pH 7 (PP3), 1% proteose peptone no. 1 at pH 7, phosphate-buffered saline, or 15 ppt artificial seawater (ASW) (Instant Ocean; Fisher Scientific). Cultures were incubated statically at 37°C and sampled on days 1, 2, 3, 7, and 10 postinoculation for the presence of altered colony types in serially diluted cultures that were spread plated to LA. Two independent experiments were performed using triplicate flasks in each. At least 80 colonies were observed for plate counts from each flask. The percentage of each Op colony type versus Tr was calculated as a function of the total colony number and based on the average of the plate counts for the two independent experiments for each strain. When available, isolated Tr colonies from each time point in these experiments were frozen in LB with 50% glycerol at -70°C. The Tr isolates from frozen stock were inoculated as described above into either PP3 or LB and incubated statically at 37°C. Cultures were sampled at 24 h for colony types as described above.

DNA cloning and sequencing. The group 1 CPS operon of *V. vulnificus* MO6-24/O was recovered by a combination of transposon marker-assisted cloning and PCR amplification, using primers that were previously described (62) or were derived from DNA sequence provided by Paul Gulig (unpublished data). The DNA was extracted using a DNeasy kit (QIAGEN), and templates (100 ng/25-µl reaction mixture) were amplified by PCR with *Pfu* DNA Polymerase (Stratagene) or the Expand Long Template PCR System (Roche) for longer amplicons. PCRs were performed on a thermocycler (GeneAmp PCR System 2400, Perkin-Elmer, or Mastercyclergradient, Eppendorf) under the following conditions: incubation at 94°C for 5 min and 25 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min with a final 7-min extension at 72°C or, for longer amplicons, according to the manufacturer's instructions (Expand Long; Roche). Primer sets included orf1F (5'-CCAGCAACTTACGTTCACTT-3') and wzcR1 (5'-CTGCGCGCTACATCAATCTCTT-3'), wzcF1 (5'-AAGAGATTGATG TAGCGCGCAG-3') and wbjBR (5'-GCTCAGGTATATGATGAGCA-3'), and wbjBF (5'-GCTCATCATATACCTGAGC-3') and wbfVR (5'-CGATTTGAG ACTATACAGTCCG-3'). Amplicons were purified using either Ultrafree-MC Cen-

TABLE 2. Transitions in colony type for opaque *V. vulnificus* strains

Strain	Transitions of Op to Tr or sectored colonies (%) at indicated time postinoculation (days) ^a				
	1	2	3	7	10
MO6-24/O	0.7 ± 0.8	7.1 ± 2.3	40.5 ± 15.1	64.0 ± 13.9	72.6 ± 15.7
LC4/O	0.3 ± 0.4	3.3 ± 3.6	16.0 ± 0.2	22.7 ± 15.2	46.5 ± 6.8
C7184/O	0.0 ± 0.0	20.3 ± 3.0	37.9 ± 0.5	49.3 ± 2.8	68.4 ± 17.6
E4125/O					
Tr	7.9 ± 5.5	14.9 ± 8.6	23.4 ± 11.2	76.5 ± 26.8	32.8 ± 12.2
Sec	15.0 ± 4.9	29.4 ± 22.1	19.4 ± 10.6	13.5 ± 18.4	47.9 ± 32.3
CMCP6/O	1.1 ± 1.2	3.4 ± 3.0	4.7 ± 2.2 ^b	53.0 ± 47.2	59.5 ± 42.1
YJ016/O	0.2 ± 0.2	1.1 ± 0.3	4.8 ± 5.1 ^b	4.2 ± 2.4 ^b	20.3 ± 19.2

^a Op isolates were inoculated into PP3 medium as described in the text and plated on LA to observe changes in colony morphology over time. Percentages of Tr colonies as a fraction of total colony number are shown for all strains, and percentages of Tr and sectored (Sec) colonies are shown for E4125/O.

^b Significant ($P < 0.01$) differences in the percentages of altered colony types among strains were noted for YJ016 and CMCP6 versus C7184 and MO6-24 at 3 days and for YJ016 versus C7184, CMCP6, and MO6-24 at 7 days.

trifugal Filter Devices (Millipore) or a GeneClean system (Q-BIOgene). Automated DNA cycle sequencing (Applied Biosystems) of plasmid clones and PCR amplifications was performed by the University of Florida ICBR core facility. Nucleotide sequence identity searches and alignments were done with the TFASTA, FASTA, or PILEUP program (GCG Wisconsin Package) or BLAST (National Center for Biotechnology Information).

Distribution of CPS alleles and genotypes. Strains of *V. vulnificus* were examined for specific CPS alleles by PCR screening using a common forward primer (wzaF2 [5'-CGATGGAATCGTGTGATCAGT-3']) and a reverse primer that was either HP1 specific (hpR1 [5'-TCGCGTTATCTGATCAACCA-3']) or HP2 specific (hpR2 [5'-GAACCTTCTGCGATGTTTGATGG-3']). CPS operons from both alleles were also examined for genetic variation at this locus following phase induction using PCR. Op ($n = 1$) or Tr ($n \geq 100$) isolates from each strain were obtained from days 3 and 7 of the phase induction studies described above. Crude DNA extracts were prepared from the supernatants of washed (once in PBS) and boiled (10 min) cultures (1 ml) that were centrifuged ($16,110 \times g$) for 10 min. DNA that spanned the conserved transport genes (*wza-wzb-wzc*) was amplified by PCR using the conditions described above and either primers wzaF1 (5'-GACGATTCAGCAGGCTCTTA-3') and wzcR2 (5'-TCCATCATCGCAAAATGCAAGCTG-3') or wzaF2 (5'-CGATGGAATCGTGTGATCAGT-3') and wzcR3 (5'-CAGCACCCTAAGGTATGCTTC-3'). Cultures that did not amplify by PCR at this locus were also analyzed for the presence of individual genes using primers derived from either *wza* (wzaF1 and wzaR [5'-TCGCGTTATCTGATCAACCA-3']), *wzb* (wzbF [5'-GGTTGATCAGATAACGCGAA-3'] and wzbR [5'-AAGGAATACAAGCGTCTAGG-3']), *wzc* (wzcF2 [5'-CCCGGAAATGAACGAGACAATG-3'] and wzcR2), or *wbV* (wbVF [5'-CTATCGTAGATGTGGATTTGAG-3'] and wbFVR). Additionally, *wvhA* primers were used to test the stability of chromosomal DNA, using PCR conditions as previously described (6).

Complementation of *wzb_v* deletion. The *wzb_v* gene was amplified by PCR with wzbF and wzbR from both MO6-24/O and LC4/O templates, yielding a 1-kb product. Cloning vector pGEMT-Easy (Promega) was used for the 1-kb amplicon that was transformed in *E. coli* JM109 (Promega) competent cells. An EcoRI-digested fragment containing the *wzb_v* deletion was further subcloned into a broad-host-range vector, pRK404 (10), in conjugation-competent *E. coli* S17-1 λpir (48) to yield pWZB. Recombinant DNA was introduced into Tr *V. vulnificus* through conjugation as previously described (62). Transconjugants were plated on LA plates containing tetracycline and polymyxin, and complemented strains were selected by reversion to Op colony morphology.

Electron microscopy. *V. vulnificus* cells were recovered from overnight growth on LA plates and negatively stained with osmium vapors (OsO₄, 4%; Ted Pella Inc., Redding, CA). Copper microscopy grids (300 mesh; Ted Pella Inc., Redding, CA) were placed on a microscope slide with a drop of LB. A loopful of the bacterial culture was removed from each plate and used to inoculate the broth droplets. The grids were placed in a chamber with OsO₄ vapors for 45 min to fix and negatively stain the samples. Lastly, the grids were washed gently with water and dried for 10 to 15 min before visualization by transmission electron microscopy (EM-10; Zeiss, CA).

Statistical analysis. Statistical significances were determined by an analysis of variance on transformed data in SAS 8.0. Also, Student's *t* test (one-tail distribution with unequal variance) and chi-squared analysis were performed in Microsoft Excel.

Nucleotide sequence accession numbers. The *V. vulnificus* MO6-24/O CPS operon sequence, the entire group 1 CPS operon has been submitted to GenBank (DQ360502).

RESULTS

Induction of Op/Tr phase transitions. Virulence is influenced by phase variation in *V. vulnificus*, and reversible transitions between Op/virulent and Tr/avirulent colonies were previously observed in LB (64). However, this process was not investigated experimentally. Therefore, kinetics of phase variation were examined in triplicate experiments using media previously reported to increase the appearance of altered colony types in *V. cholerae* (1). Inocula were derived from individual Op or Tr colonies and cultured in broth medium prepared with PP3 at pH 7 instead of the alkaline pH 8 that was used in the *V. cholerae* study. Cultures were scored for altered colony types by spread plating them to LA. As shown in Table 2, all Op strains showed Tr colonies after 2 days of incubation. The percentage of Tr colonies in PP3 broth culture increased from <1.0% to >50% in most strains with extended incubation by the end of the experiment. The increase in the number of Tr cells was generally equivalent to the decrease in Op cells, indicating that a "switching" had occurred that could not be attributed simply to die off of Op cells or growth of Tr cells. Also, extensive cell division of Tr cells would be unlikely under the stationary-phase conditions used in these studies. Op-to-Tr conversion was not observed in medium other than PP3, including LB or proteose peptone no. 1 broth, PBS, or ASW. The stability of the Op phenotype varied among strains, as evidenced by differences in transition rates to the avirulent Tr phenotype. For example, YJ016/O produced Tr colonies at significantly ($P < 0.01$) lower frequency than other strains and did not exceed 5% until day 10. The appearance of Tr colonies in CMCP6 was delayed but exceeded 50% by day 7. Interestingly, *V. vulnificus* E4125 was extremely unstable and produced sectored colonies, as well as Op and Tr morphotypes. Sectored colonies were a mixture of Op and Tr phenotypes in a single colony, indicating a highly variable population on solid medium. In general, the transitions to Tr phenotype increased with time; however, greater variability was noted with extended incubation, as shown by the larger standard deviations at later time points.

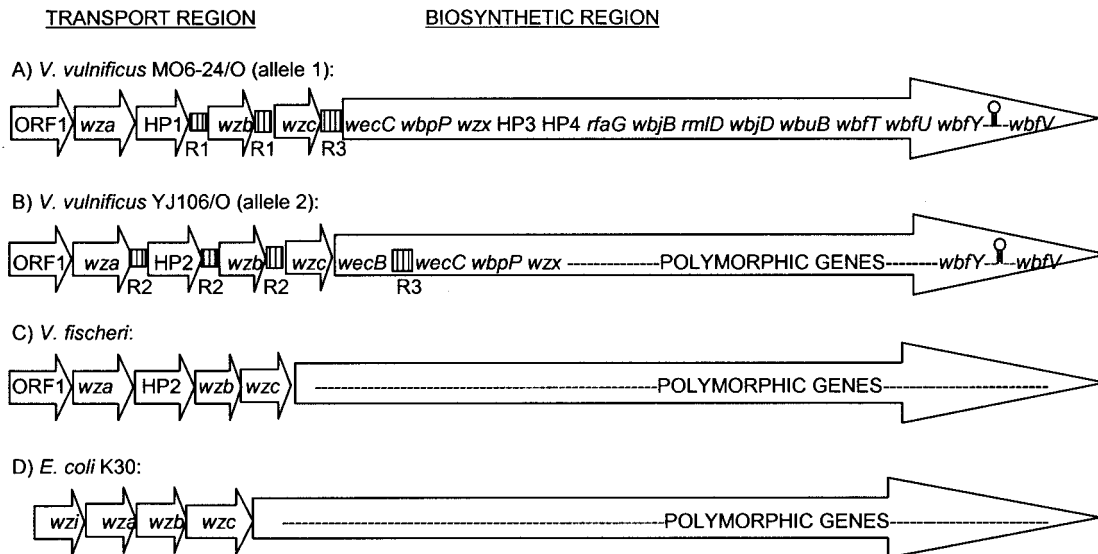


FIG. 1. Genetic organization of group 1 CPS operons. Group 1 CPS operon structure includes conserved transport and polymorphic biosynthetic regions for (A) *V. vulnificus* MO6-24/O (allele 1), (B) *V. vulnificus* YJ016 (allele 2), (C) *V. fischeri*, and (D) *E. coli* K30. The directions of transcription are indicated by arrows, and the predicted stem-loop transcriptional terminator is shown for *V. vulnificus* operons.

Compared to Op isolates, Tr phenotypes were extremely stable in PP3 broth and never reverted to Op. However, two distinct phenotypes were observed in LB culture for Tr isolates that were derived from originally opaque strains after extended incubation in PP3. Some Tr isolates now appeared “hypervariable” in LB and produced $48.9\% \pm 9.1\%$ Op colonies within 24 h postinoculation, compared to the 3 to 7 days required to achieve similar levels of Op-to-Tr transitions. Hypervariability was also indicated by the appearance of sectored colonies, which were $5.9\% \pm 1.2\%$ of the total population. Hypervariability was observed only after subculture in LB, and continued passage in PP3 negated the appearance of this phenotype. On the other hand, other Tr isolates were locked in the translucent phase and did not revert to Op in LB. These results demonstrated that at least some of the Tr isolates maintained reversible phase variation and that the stability of the CPS phenotypes was responsive to different growth conditions.

Analysis of the *V. vulnificus* group 1 CPS operon. The genetic basis for Op/Tr phase variation is unknown in *V. vulnificus*. Mutations within the group 1 CPS operon were previously noted by Southern analysis of phase variants (62). We hypothesized that genetic diversity at this locus may account for the strain differences in phase variation described above. As shown in Fig. 1, the genetic organization of the *V. vulnificus* MO6-24/O CPS operon resembled group 1 CPS operons originally described in *E. coli* and other *Enterobacteriaceae* (61). Group 1 operons are usually expressed as a single transcript of about 15 to 20 kb, and proposed limits for the *V. vulnificus* group 1 operon included a putative promoter located just upstream of the first open reading frame, ORF1 (the -10 site is located 30 bp upstream of ORF1) (62), and a Rho-independent transcriptional terminator with a typical stem-loop structure (-19.4 kcal/mol at 37°C) at the terminus of the polymorphic region of the operon (24).

The first open reading frame (ORF1) in the *V. vulnificus*

group 1 operon showed no homology to sequences in GenBank but was followed by conserved CPS genes (*wza-wzb-wzc*) required for translocation and expression on the cell surface in *E. coli*. The *V. vulnificus* Wza had 61% deduced amino acid identity to *E. coli* (78% similarity; NP_415503.1), Wzb had 55% amino acid identity (75% similarity; AAD21563.1), and Wzc had 47% amino acid identity (69% similarity; AAD21564.1). The two *V. vulnificus* genomes available in GenBank from either YJ016 (NC_005139 [7]) or CMCP6 (NC_004459; direct submission to GenBank) were also similar in structure to the MO6-24/O group 1 operon reported here. *Vibrio fischeri* (NC_006840 [45]) and *Vibrio parahaemolyticus* (NC_004605 [26]) genomes also shared homologous operons, but homologues were not found in *V. cholerae*. When present, these operons are located on the larger of the two chromosomes in all *Vibrio* spp.; however, Chen et al. (7) described *wza* and *wzc* sequences that were located outside the operon on the small chromosome. Second copies of the *wza*, *wzb*, and *wzc* genes (*wza22 min*, *wzb22 min*, and *wzc22 min*) were also described in *E. coli* K30 and K-12 strains, and these gene products participate with low efficiency in K30 CPS and colanic acid production (58, 66). Unlike gene copies in *E. coli*, which demonstrated $>50\%$ deduced amino acid identity, the second “homologues” in *V. vulnificus* showed relatively little deduced amino acid identity ($<25\%$) and no DNA identity. Thus, these second loci in *V. vulnificus* are unlikely to be group 1 homologues, as they lacked the genetic conservation that is characteristic of group 1 operons.

The conserved group 1 transport genes were followed by polymorphic genes for CPS biosynthesis, which varied greatly among *V. vulnificus* strains and which determine the diverse polysaccharide structure characteristic of this species. All ORFs following the conserved *wza-wzb-wzc* genes in the *V. vulnificus* group 1 CPS operon exhibited similarity to genes related to biosynthesis of either CPS or LPS. The repeating

units of the *V. vulnificus* MO6-24/O CPS polymer are composed of three *N*-acetyl-quinovosamine (QuiNAc) and one *N*-acetyl-galactosamine uronic acid (GalNAcA) sugar residues (41). Uronic acid sugars are common to CPS structures among strains of this species (5). Both QuiNAc and GalNAcA have also been identified in O-specific chains of LPS of *Pseudomonas aeruginosa* strains (22). Not surprisingly, many of the *V. vulnificus* genes showed the closest homology to genes involved in the *Pseudomonas* LPS biosynthetic pathway. The structural configurations of the sugar moieties of *V. cholerae* O139 LPS and CPS are identical and contain the trisaccharide GlcNAc-GalA-QuiNAc (59, 67). The last four genes (*wbfT_{Vv}*, *wbfU_{Vv}*, *wbfY_{Vv}*, and *wbfV_{Vv}*) in the *V. vulnificus* MO6-24/O operon had the closest deduced amino acid homology (66 to 81% identity) to proteins required for polysaccharide biosynthesis in the *V. cholerae* O139 CPS/LPS operon (67). We also found homologues for *wbfY* and *wbfV* in both of the published *V. vulnificus* genomic sequences. Additionally, the CMCP6 genome contained a *wbfU_{Vv}* homologue. The Rho-independent termination stem-loop structure was found immediately upstream of *wbfV*, indicating that this gene is not part of the group 1 operon in *V. vulnificus*.

Genetic (allelic) variation in the *V. vulnificus* group 1 CPS operon. As shown in Fig. 1, *V. vulnificus* strains MO6-24/O and YJ016/O exhibited heterogeneity within the CPS operon sequences. Two distinct genetic profiles were observed at the same locus in the different strains and are referred to as allele 1 and allele 2. Allele 1 was also found in CMCP6 (not shown), and it diverged from allele 2 at sequences encoding hypothetical proteins (HP1 and HP2, respectively) and at repetitive elements (R1 and R2, respectively). To further examine the distribution of CPS alleles within the species, allele-specific PCR products were compared for clinical versus environmental strains. Interestingly, the allelic distribution varied with strain origin ($P < 0.0001$; chi-squared test). As shown in Table 1, the CPS allele 1 was more common among clinical (87%) than environmental strains, while allele 2 was more frequent in environmental (87%) than clinical strains. Alignment of DNA sequences from multiple amplicons showed that both the gene sequence and location were highly conserved within a particular allele. For example, HP1 DNA sequences were $\geq 98\%$ identical within the allele and showed similar placement in the operon (Fig. 2A). Similar conservation of HP2 sequence and structure was observed within allele 2 (Fig. 2B). Conversely, no DNA sequence identity and only 43% deduced amino acid identity (66% similarity) were observed between HP1 and HP2; however, both showed the closest homology to a conserved domain of the 23S ribosomal protein (CDD23859), with the closest homologues in two different species of *Shewanella* (ZP_00587012.1 [*Shewanella amazonensis*] and ZP_00634125.1 [*Shewanella denitrificans*], respectively). HP segments were inserted between the *wza* and *wzb* genes in both *V. vulnificus* and *V. fischeri* but were absent in all other species, including *V. parahaemolyticus* (not shown). HP sequence in *V. fischeri* showed 82% nucleotide identity and 57% deduced amino acid identity (77% similarity) to *V. vulnificus* HP2.

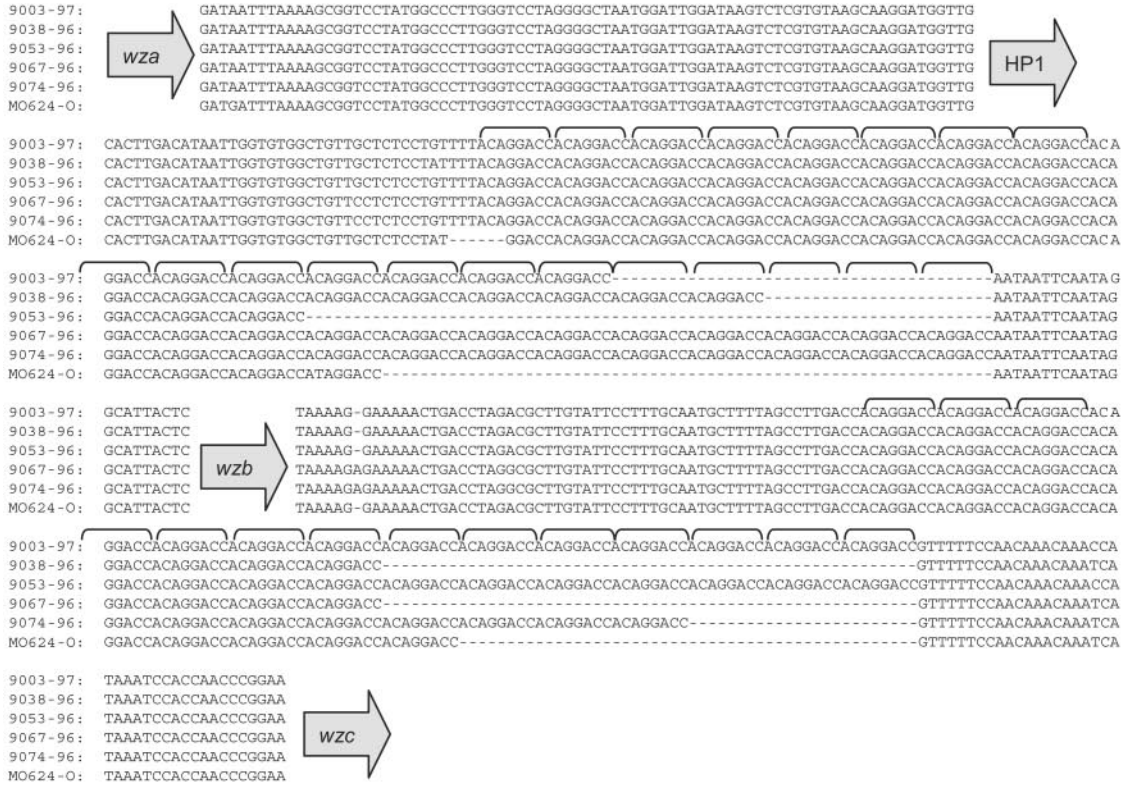
HP sequences in *V. vulnificus* were linked to paired tandem cassettes of directly repeated DNA elements. Allelic variation was also observed in repetitive elements and included differences in DNA sequence and location within the operon. For

example, repetitive elements in allele 1 immediately flanked the *wzb* gene, but an additional element was located just downstream of *wza* in allele 2 (Fig. 1). In allele 1, multiple copies of the same 8-bp (ACAGGACC) segment were sequentially and directly repeated within the repetitive elements. On the other hand, repetitive subunits in allele 2 were more variable (A/CC TAGG/AAA/C) in sequence than those in allele 1. In both alleles, these elements localized only to group 1 CPS operons and were not seen elsewhere in the genomes of either of the two published *V. vulnificus* sequences. However, a downstream repetitive element (R3) associated with the group 1 operon was also found dispersed at multiple ($n = 5$) divergent sites throughout both genomic sequences that contained the alternate alleles. This element resembled R1 with multicopy repeated subunits but differed in the primary subunit length (7 bp) and sequence (CTAGAAC). Repetitive elements were also described upstream of LPS and CPS operons of *V. cholerae* (67) and were identical to the *V. vulnificus* R3 repeated sequences. However, group 1 CPS operons of other species do not contain repetitive elements, and this feature was unique to the species.

Multiple genotypes determine the Tr phenotype. In order to examine the relationship of the CPS operon to phase variation, genetic organization was examined in Tr isolates from the phase induction studies described above. Op, Tr, or sectored isolates were screened for divergent genotypes using PCR primers that spanned the *wza-wzb-wzc* region (3.7 kb). PCR amplicons for representative strains are shown in Fig. 3. Op strains always produced the 3.7-kb band; however, Tr isolates showed genetic variants that were designated either TR1, TR2, TR2A, or TR3 genotype. TR1 variants also produced the 3.7-kb band equivalent to the Op strains, TR2 and TR2A showed smaller single amplicons (3.3 and 3.0 kb, respectively) than the Op strains, and TR3 failed to amplify by PCR. The entire group 1 operon for an MO6-24/TR1 isolate was sequenced, and all TR1 genes were 100% identical in sequence and gene order to the Op parent CPS operon. The sequences of *wza-wzb-wzc* PCR products from multiple TR1 isolates ($n = 3$) also confirmed that there were no mutations at this locus. These results indicated that the TR1 operons were intact and that mutations in the primary DNA sequence did not account for the Tr phenotype in these isolates. On the other hand, the DNA sequences of TR2 and TR2A isolates confirmed deletion mutations and showed that the entire 435-bp *wzb* gene was absent in both genotypes and that HP2 was also deleted in TR2A. Deletions for TR2 and TR2A included bp 2754 to 3323 (DQ360502) and bp 345279 to 346423 (NC_005139), respectively.

No DNA sequence was available for TR3 because these isolates failed to amplify by PCR. The lack of a PCR product spanning the *wza_{Vv}*-to-*wzc_{Vv}* region suggested that a deletion had occurred that extended beyond the *wzb* gene. Additional primer sets that individually targeted each of the transport genes (*wza_{Vv}*, *wzb_{Vv}*, *wzc_{Vv}*) also did not amplify TR3 by PCR but did amplify sequences in Op variants of the same strain. A conserved gene, *wbfV_{Vv}*, found just downstream of the CPS operons (Fig. 1) in both the published genomes and in *V. vulnificus* MO6-24/O in the present study, was also targeted by PCR. Primers derived from this sequence produced PCR products in opaque variants of all strains but again failed to yield PCR products from TR3 isolates. Additional primer sets

A) Allele 1



B) Allele 2

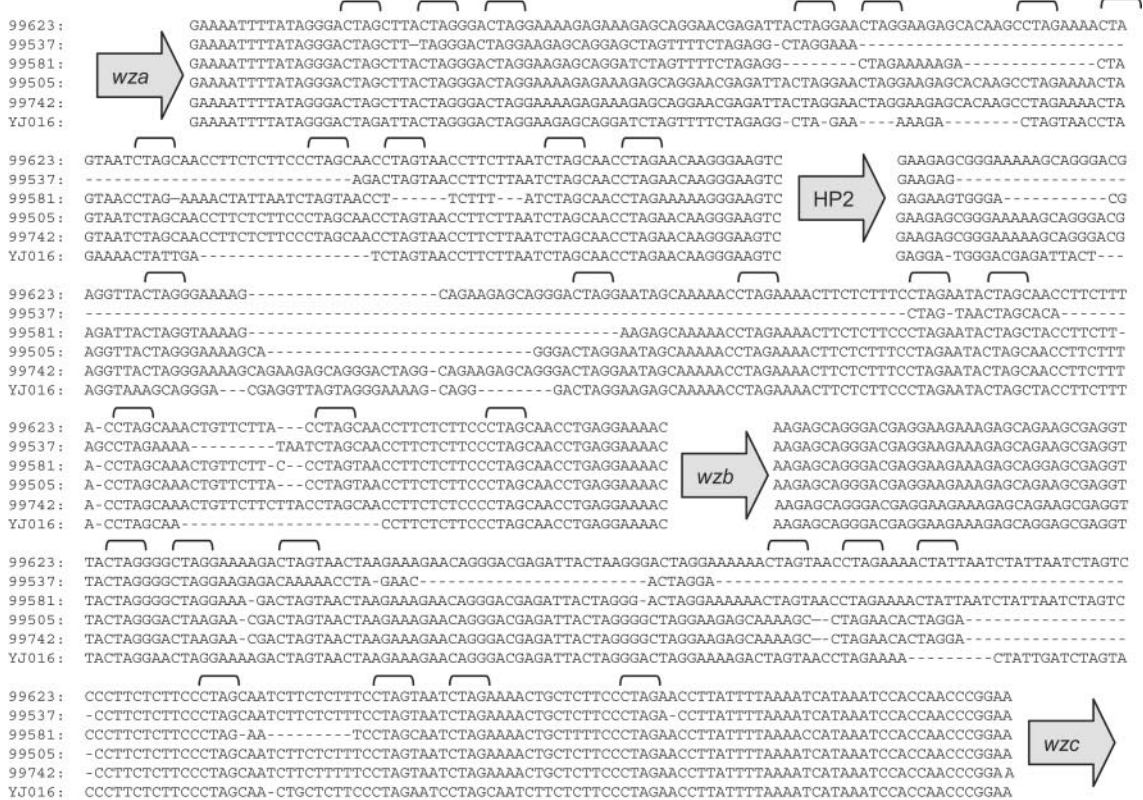


FIG. 2. Alignment of group 1 CPS alleles for *V. vulnificus*. DNA alignment of the conserved transport regions are shown for (A) allele 1 and (B) allele 2 of the group 1 CPS operon for multiple strains. The strain numbers refer to CDC strain designations listed in Table 1. The brackets indicate multicopy R1 identical repeats (ACAGGACC) in allele 1 or R2 variable repeats (A/CCTAGAG/AGAA/C) in allele 2.

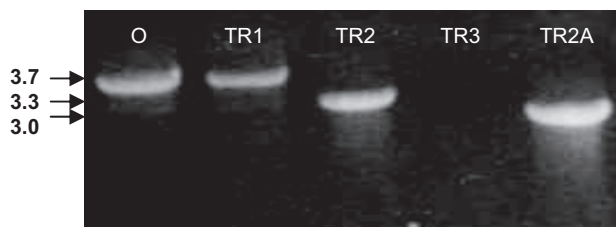


FIG. 3. PCR analysis of CPS transport region in *V. vulnificus* phase variants. PCR amplicons derived from primers that spanned the transport region (*wza* to *wzc*) of the CPS operon, as described in Materials and Methods, are shown for phase variants of *V. vulnificus* MO6-24/O, MO6-24/TR1 (TR1), LC4/TR2 (TR2), 345/TR3 (TR3), and YJ016/TR2A (TR2A). Approximate amplicon sizes (arrows) are derived from DNA standards (not shown).

at 3 and 7 kb upstream of ORF1 showed variable results among TR3 isolates of different strains (data not shown), and therefore, the limits of these deletions are likely to be highly variable. However, species-specific PCR primers (6) for the *vvhA* hemolysin gene produced identical products in all TR3 strains (not shown). These results confirmed that DNA deletions in the CPS operon extended beyond *wzb* excisions in TR3.

Allelic variation and deletion mutations. In the phase induction studies described above, strains with CPS allele 1 showed significantly higher frequencies of Op-to-Tr transitions than those with allele 2, suggesting a possible correlation between the CPS allele and the stability of the opaque phenotype. We hypothesized that differences in the accumulation of deletion mutants might contribute to the relative frequency of Op-to-Tr transitions, as these mutations represent an irreversible and stable phenotype. Therefore, the distributions of different genotypes (TR1, TR2, TR2A, and TR3) were sampled among strains with either allele 1 or allele 2. To maximize sample size and reduce variation due to strain differences, Tr isolates were recovered from PP3 broth on days 3 and 7 postinoculation in two independent experiments ($n \geq 100$ isolates/strain for each experiment). The Op genotype was inferred from colony morphology, and Tr genotypes were determined by PCR amplification. As shown in Table 3, no significant differences in cumulative Op versus Tr colony types were observed between alleles. The TR1 genotype (indicating intact CPS operons) was

seen in all strains, but the distribution of TR2 deletions was greater among strains with CPS allele 1 (ranging from 1.8 to 27.9%) than among allele 2 strains (0 to 1.0%) and averaged 12.1% versus 0.3%, respectively. TR3 mutations (no PCR product) were seen infrequently in allele 1 but not at all in allele 2. Also, the TR2A genotype was observed only in allele 2 and was never observed in the >400 allele 1 isolates examined. Overall, total-deletion mutations (TR2 plus TR2A plus TR3) were significantly ($P < 0.003$ by *t* test) greater in strains with allele 1 than in those with allele 2. Interestingly, differences within allele 1 for the percentage of TR2 mutations roughly corresponded ($R^2 = 0.74$) to the ratio of the repetitive-subunit copy number for upstream versus downstream elements (Table 3). For example, CMCP6, with the lowest ratio (0.9), showed the lowest distribution of deletion mutations compared to strains with higher ratios (2.3 or 3.1); however, this relationship remains speculative.

These results demonstrated that the type of deletion mutation was dependent upon allelic differences in the placement of repetitive elements. For example, TR2 mutations with excision of only *wzb* were observed in both alleles where repetitive elements flanked this site (Fig. 4). However, only allele 2 produced the TR2A deletion, and excision of both *wzb* and HP2 sequences corresponded to the placement of the repeated element upstream of HP2 in allele 2. DNA sequences from multiple isolates ($n = 3$) were consistent for each type of deletion. Surrounding DNA sequences for the promoter region, ORF1, *wza*, and *wzc* remained intact and also contained remnants of repetitive DNA, but always with reduced total copy numbers. Thus, the extent of the deletion mutation corresponded to the placement of repetitive elements at the site, and strain differences in deletion mutations were determined by alternate CPS alleles.

Phenotypic characterization of Tr genotypes. We previously examined *V. vulnificus* MO6-24/T, a translucent isolate that was identified as the TR1 genotype in the present study, and determined that this strain expressed residual capsule on the surface but in reduced amounts compared to the Op parent strain (63). In the prior report, the TR1 strain was examined in detail for CPS expression by immunoelectron microscopy and quantitative flow cytometry. The amount of surface CPS expressed in this strain was intermediate between the Op parent

TABLE 3. Allelic variation in distribution of deletion mutations

<i>V. vulnificus</i> strain	Cumulative genotype distribution (%) ^a					Ratio ^b (copy no.)
	Op	TR1	TR2	TR2A	TR3	
Allele 1						
MO6-24	41.1 ± 1.7	25.5 ± 5.0	27.9 ± 0.0	0	4.8 ± 6.7	3.1
LC4	81.0 ± 5.4	6.3 ± 5.3	12.7 ± 0.1	0	0	2.3
C7184	56.9 ± 5.6	37.2 ± 6.6	5.9 ± 1.0	0	0	2.3
CMCP6	85.9 ± 2.0	11.5 ± 2.6	1.8 ± 2.5	0	0.9 ± 0.8	0.9
Allele 2						
YJ016	96.1 ± 3.5	3.4 ± 0.2	0	0.5 ± 0.1	0	NA
99-537DP-G7	68.8 ± 14.9	30.6 ± 15.8	1.0 ± 1.1	0	0	NA
99-623DP-F5	49.5 ± 36.9	50.5 ± 36.9	0	0	0	NA

^a Cumulative genotype distribution indicates the percentage of the total colonies recovered from PP3 broth as an average of two experiments ± standard deviation. The Op genotype was inferred from colony morphology, and the distribution of Tr genotypes was based on PCR screening. Cultures were sampled on days 3 and 7 postinoculation to obtain a combined total of at least 100 Tr isolates for evaluation.

^b Copy number ratio refers to the number of repeated subunits within allele 1 for upstream-downstream repetitive elements. NA, not applicable.

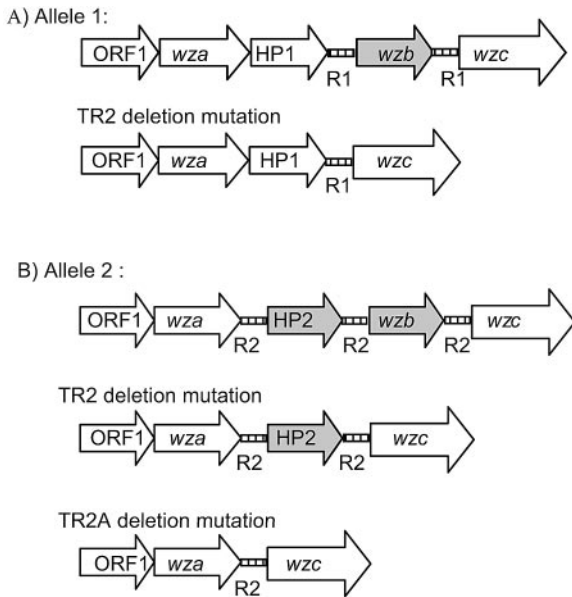


FIG. 4. Allelic variation and deletion mutations in the group 1 CPS operon. Group 1 CPS operon structure and corresponding TR2 and TR2A deletion mutants are shown for (A) allele 1 and (B) allele 2. The directions of transcription are indicated by arrows, and genes removed by deletion mutations are shaded.

strain and acapsular mutants with either specific inactivation of the *wza* gene or polar *TnphoA* insertion into the CPS operon (63, 64). Furthermore, Yoshida et al. (69) also observed Tr variants that were either partially encapsulated or acapsular by electron microscopy. Therefore, Tr isolates with different genotypes were examined for CPS expression using negative staining and electron microscopy (Table 4). The observations were consistent with the previous descriptions, and reduced CPS expression was confirmed in TR1 strains. However, TR2 mutants were acapsular and were identical in appearance to acapsular mutants with insertional inactivation of *wza*_{Δv} (not shown).

As described for the phase induction experiments above, some of the Tr strains obtained from passage of Op strains in PP3 showed reversible phase variation to the Op phenotype after incubation in LB, while others did not. PCR analysis of Tr genotypes showed that phase variability was observed only in isolates ($n = 24$) with the TR1 genotype. The strains ($n = 24$) that were stable in the Tr phenotype were deletion mutants with the TR2, TR2A, or TR3 genotype. Thus, deletion mutants were locked into the translucent phase, presumably due to the irreversible loss of *wzb* or other genes. To confirm that the TR2 deletions were specific to the *V. vulnificus* *wzb* gene and responsible for the shift to the Tr phenotype, MO6-24/TR2 and LC4/TR2 were complemented in *trans* from plasmid constructs of *wzb* derived from either MO6-24/O (pWZBM) or LC4/O (pWZBL) DNA. Introduction of either plasmid into the deletion mutant yielded Op colonies, indicating this gene was able to repair the defect. Recovered CPS expression appeared equivalent to that of the Op parent strain by electron microscopy (Table 4), but TR2 with vector controls remained acapsular (not shown). TR2A strains included deletions of both *wzb* and HP2; however, complementation with *wzb* in *trans* also

restored the Op phenotype for this mutant and further demonstrated that *wzb* was required for CPS expression while HP2 was not. Discrepancies in the literature regarding CPS expression of differences in associated Tr genotypes. In summary, the genetic basis for transition to the Tr avirulent phenotype in *V. vulnificus* involves at least two types of genetic alterations: (i) reversible phase variants that do not involve mutations of the group 1 CPS operon and (ii) mutants with site-specific deletion of *wzb* in the group 1 CPS operon.

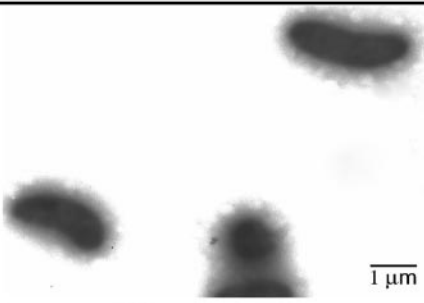

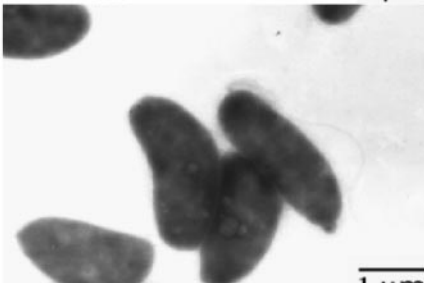
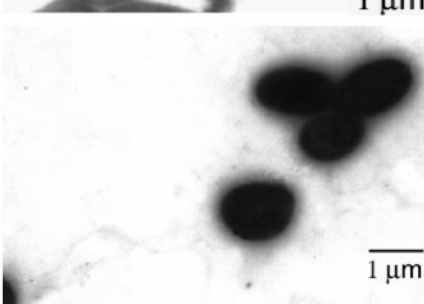
DISCUSSION

These results provide the first experimental analysis of *V. vulnificus* CPS “phase variation.” Transitions between colony types in *V. vulnificus* varied greatly among strains and were dependent upon the initial colony genotype and culture conditions. For example, incubation in specific medium (PP3) produced Op-to-Tr but not Tr-to-Op transition. This response occurred with extended incubation during late stationary phase; however, some Tr strains derived from PP3 became “hypervariable” and produced Op or sectored colonies with overnight incubation in LB. We speculate that these variable responses and the greater standard deviation observed during later stages of incubation are the consequence of mutations that accumulate during stationary phase. Furthermore, genetic examination of phase variants showed that multiple types of DNA mutations were involved in the transition of the Op to the Tr phenotype.

Genetic events related to Op-to-Tr transitions resulted in at least three possible outcomes: reversible TR1 phase variants with intermediate CPS expression and intact CPS operons, TR2 acapsular mutants with site-specific deletions of the *wzb* gene of the group 1 CPS operon, and TR3 acapsular mutants with more extensive deletions in the CPS operon. As expected, deletion mutants were always stable and did not revert to the Op phenotype. The CPS operon in TR1 isolates was intact; therefore, the down-regulation of CPS expression in these strains must involve mutations at loci outside the CPS locus. Multiple regulons are described for regulation of CPS in *V. parahaemolyticus* (15). Deletion mutations in *opaR*, a regulatory gene and a *luxR* homologue, were shown to determine Op-to-Tr transitions in *V. parahaemolyticus* and to reduce CPS expression (28). Conversely, a frameshift mutation in a *V. cholerae* *opaR* homologue (*hapR*) determines transition to the rugose phenotype with increased CPS gene expression (68). Thus, these regulons have opposite functions with regard to CPS expression in the different species and highlight the complexity of CPS regulation. The *opaR/hapR* homologue in *V. vulnificus* is the *smcR* gene, which positively regulates starvation response, protease expression, and biofilm formation (30). However, prior research (47) showed that the *smcR* mutation in *V. vulnificus* does not alter the colony type, group 1 CPS-dependent virulence in mice, or resistance to the bactericidal effects of serum. These results led to the conclusion that *smcR* does not regulate group 1 CPS.

Repetitive sequences in other species generally mediate phase variation through localized recombination, leading to insertions and deletions within the repetitive element that produce intragenic frameshifts or promoter dysfunction (20).

TABLE 4. Phenotypic characterization of *V. vulnificus* genotypes

Strain ^a	Phase variable	Description	Electron micrograph ^b
O	Yes	Op colony morphology Intact CPS operon Phase variable	
TR1	Yes	Tr colony morphology Intact CPS operon Phase variable	
TR2	No	Tr colony morphology Deletion of <i>wzb</i> Phase stable	
TR2 (pWZB)	No	Op colony morphology TR2 complemented <i>in trans</i> with <i>wzb</i> on plasmid (pWZB)	
TR3	No	Tr colony morphology Multiple deletions Phase stable	Not shown

^aMO6-24/O (O), Tr variants (TR1, TR2, and TR3), and complemented TR2 deletion mutant [TR2(pWZB)] are shown.

^b Electron micrographs of negatively stained cells, as described in Materials and Methods.

However, the intergenic location of the *V. vulnificus* repetitive element would not produce frameshift mutations. Genetic rearrangements related to phase-variable events are generally repairable mutations mediated by DNA repair enzymes, enzyme-regulated genetic inversions (8), or insertion/deletion of transposons (36). Smith and Siebeling (50) identified an insertion element associated with alterations in CPS genes and

expression, but this element was not linked to the group 1 CPS operons in genomic analyses. Thus, the genetic basis for reversible phase variation in *V. vulnificus*, or any other *Vibrio* sp. for that matter, remains elusive.

The location of intergenic repetitive elements in *V. vulnificus* corresponded exactly to the site of excised DNA in deletion mutants, suggesting that these elements provide sites for ho-

mologous recombination. Direct repeats mediate deletion of intervening sequence in the gonococcal *pilE* locus, but these repetitive elements are intragenic and do not remove the entire gene (2, 18). Similar deletion mutations resulting in gene excision were previously noted for the *bexA* gene of the *Haemophilus influenzae* CPS operon; however, this mutation involved recombination between flanking *cap* genes rather than intergenic repetitive elements (for a review, see reference 43). An alternative function for repetitive elements is site-specific recognition by DNA binding enzymes for DNA excision and repair. For example, invertases recognize inverted repeats and control CPS expression in *Bacteriodes* through reversible inversion of promoter regions of related operons (8). More extensive insertions/deletions are referred to as indels and are also associated with repetitive elements (4, 56). Comparative genomics revealed that indels contribute to the evolution of bacterial pathogenesis (27) and symbiosis (35) in many species, including *Vibrio* spp. (55). *V. vulnificus* deletions did not show reversible phase variation in broth culture, but deletion mutants retained genes required for CPS biosynthesis, as well as remnants of repetitive DNA elements that may serve as insertion sites for homologous recombination leading to the recovery of deleted DNA (18). Therefore, excised genetic material could possibly be recovered from exogenous sources through transducing phage, especially in mixed communities under extreme selective pressures imposed by host immune responses or volatile conditions in estuarine environments. Recombinant constructs bearing alternate repetitive-subunit copy numbers and/or sequences in a constant genetic background will be needed to confirm the mechanisms of deletion mutation in *V. vulnificus*. In summary, the data here are consistent with the hypothesis that deletion mutations are determined by the repetitive elements.

Divergent organization of the group 1 CPS operon was observed among strains of *V. vulnificus*, and variable DNA sequences between alleles included segments encoding an HP and repetitive DNA elements. HP sequence and placement were highly conserved among strains within each allele, but no DNA homology and only 43% deduced amino acid identity was seen between alleles. Repetitive DNA elements also varied in repetitive-subunit sequence and genetic organization between alleles. This allelic variation corresponded to strain differences in the accumulation of deletion mutations. Overall, strains with allele 1 produced deletion mutations with consistently greater frequency than allele 2 strains. Repetitive elements promote recombination events, and increased mutation frequency is dependent upon the length, GC content, and secondary structure of repetitive elements (33). *V. vulnificus* allele 1 repetitive elements were octameric repeats (ACAGGA CC) in high copy number, while allele 2 sequences were non-contiguous and more variable than those of allele 1. Allele 1 repeats generated predicted stem-loop structures due to the high GC content within subunit sequences. A transcriptional attenuator divides the *E. coli* group 1 CPS operon into two regions; however, the DNA location and sequence of this site showed no homology to repetitive elements of *V. vulnificus* (40). Antiterminator activity is associated with an *ops* locus in the "JUMPstart" region (19) of many CPS operons, and *ops* homology is seen upstream of the group 1 operon in *V. vulnificus* (62). DNA structures associated with repetitive elements

in *V. vulnificus* may also interfere with transcription and serve as possible sites for CPS regulation.

Both CPS allelic types of *V. vulnificus* produced mutations that led to the deletion of the *wzb* gene, which is thought to provide cognate phosphatase activity for phosphorylation of the tyrosines within the C-terminal 17 amino acids of Wzc in *E. coli* (66). The locations of these sites in *V. vulnificus* were similar to those in *E. coli*; however, only six of seven sites were found in *V. vulnificus* Wzc. The precise role of Wzc has not been elucidated, but studies of *E. coli* supported the hypothesis that Wzc must undergo cycles of phosphorylation and dephosphorylation for K30 CPS to be expressed. This process may contribute to "volume control" of surface CPS by coordinating with other components in the assembly complex in a manner analogous to the proposed role of Wzz in the biosynthesis of O antigens (66). Thus, loss of *wzb* in *V. vulnificus* deletion mutants would presumably interfere with the phosphorylation of Wzc to turn off surface expression of CPS. In *V. vulnificus*, deletion mutants were acapsular by electron microscopy and resembled polar transposon mutants with no CPS expression on the cell surface. Complementation in *trans* restored CPS expression, which supports the hypothesis that *wzb* is required for CPS expression on the cell surface of *V. vulnificus*. Both *wzb* and HP2 sequences were deleted in TR2A; however, introduction in *trans* of *wzb* alone still restored opacity, indicating that HP2 is not required for CPS expression. The function of HP sequences is unknown, but it is interesting that the two alleles appear to have arisen from independent insertional events of somewhat related sequences into the same site and that both were associated with similarly placed but divergent repetitive elements. We speculate that the selection for these events may reside in some undetermined advantage conferred by the deletion of *wzb*, possibly related to enhanced survival in estuarine environments.

Two distinct genetic types or alleles of the group 1 CPS operon were differentially distributed among clinical versus environmental strains of *V. vulnificus*. Potential virulence in *V. cholerae* and *V. parahaemolyticus* is clearly defined by toxin-encoding genetic markers (*ctx* and *tdh*, respectively), but these distinctions are less evident in *V. vulnificus* because all clinical and environmental strains are equally virulent in animal and cell culture models of pathogenesis (9). Previous associations of allelic variation with clinical origin were noted for *V. vulnificus* loci, including the 16S rRNA gene (34), the *viuB* gene for siderophore biosynthesis (37), and another ORF with no homology to sequences in GenBank (44). Only *viuB* has demonstrated virulence association in animal models (25), but this gene product is expressed in both clinical and environmental isolates (65). Op strains with CPS allele 1 showed increased frequency of transition to the Tr phenotype compared to strains with allele 2. However, association of allele 1 with clinical origin is difficult to reconcile, as increased deletion mutation in the CPS operon would presumably reduce virulence potential in the vertebrate host. Inferences that allelic markers equate to virulence potential are unsubstantiated, as they are not supported by virulence analysis. Thus, the relationship of these alleles to virulence potential is still unknown.

Polysaccharide capsules provide protective adaptations for increased bacterial survival in aquatic habitats and within mammalian hosts. The role of CPS in the virulence of *V.*

vulnificus in mammalian hosts has been clearly demonstrated in animal models and by the fact that all clinical strains are encapsulated. However, the relationship of CPS expression to environmental survival in estuaries is less clear. Encapsulation confers selective advantage in the survival of Op *V. vulnificus* in oyster hemocytes (16); conversely, group 1 CPS expression inhibits biofilm formation (21), which is considered a survival mechanism of aquatic bacteria (23). The species is an opportunistic pathogen, and food- or waterborne transmission is not related to passage in the human host. Therefore, evolution of genetic variation in the group 1 CPS operon of *V. vulnificus* is more likely to be related to survival in the estuarine environment rather than to increased virulence potential in the human host. Genetic organization that enables rapid reversion to genotypes with reduced or absent CPS expression may function to enhance biofilm formation or to reduce energy expenditure during starvation. To date, survey of clinical versus environmental alleles is limited, and sampling methodology, seasonality, geographic distribution, changing estuarine conditions, invertebrate host preferences, and/or environmental stressors probably contribute to observations of genetically divergent populations. Systematic environmental sampling and multi-gene analyses of loci encoding determinants for virulence and/or environmental survival are needed to provide insight into the genetic requirements and evolution of *V. vulnificus* disease.

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