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Phenotypic Evaluation of Acapsular Transposon Mutants of *Vibrio vulnificus*

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Translucent, avirulent spontaneous phase variants of *Vibrio vulnificus* MO6-24 reverted back to the original opaque, encapsulated phenotype under both in vivo and in vitro conditions. Two translucent, acapsular mutants, which did not show phase variation, were constructed by using the transposon Tn*5* IS*50*$_L$::*phoA* (Tn*phoA*). Loss of capsule was accompanied by decreases in virulence, hydrophilicity, and serum resistance. The ability to utilize transferrin-bound iron for growth was lost in only one of the two unencapsulated mutants. Our data emphasize the apparent importance of capsule in the virulence of *V. vulnificus* and indicate that utilization of transferrin-bound iron is independent of encapsulation.

Vibrio vulnificus is an estuarine bacterium that has been associated with serious wound infections and with a syndrome of primary septicemia (2, 17, 18). While wound infections can occur in healthy individuals, septicemia primarily affects compromised hosts with underlying conditions, such as hemachromatosis, cirrhosis, and alcoholism, with a reported mortality rate of greater than 50%. Disease has generally been associated with the handling or ingestion of raw shellfish (9, 22). Epidemiologic studies suggest that *V. vulnificus* accounts for over 50% of all vibrio-associated illness in this country (4).

V. vulnificus displays two distinct colony morphologies, designated as opaque and translucent. Opaque strains are encapsulated, are virulent in mice, are resistant to the bactericidal activity of serum, and can utilize transferrin-bound iron for growth (10, 11, 20, 21, 26). In contrast, translucent strains are unencapsulated (or have decreased capsular material by electron microscopy), are less virulent, are serum sensitive, and cannot grow in iron-limited media, even in the presence of transferrin that is fully iron saturated. Strains have been reported to shift from an opaque to a translucent colony morphology at a frequency of ca. 10^{-4}; however, previous studies have suggested that reversion from a translucent to an opaque morphology does not occur (21, 26).

Although the phenotypic characteristics associated with each colony morphology have been described, relationships among these characteristics and their relative importance in pathogenicity are poorly defined. In this study, we isolated translucent variants of an originally opaque, virulent *V. vulnificus* strain and compared the phenotypic characteristics of these variants with the phenotypic characteristics of translucent transposon mutants of the same strain. We wanted to determine if spontaneous reversion from a translucent to an opaque morphology could occur; we were also interested in seeing if a single transposon insertion was sufficient to result in the loss of the opaque morphology and if this loss was accompanied by the loss of other virulence-associated characteristics.

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MATERIALS AND METHODS

Bacteria and media. *V. vulnificus* MO6-24, isolated from a patient with septicemia, was used in all studies. Frozen stocks were maintained at −70°C in L broth (13) containing 50% glycerol. The original isolate (designated MO6-24/O) had an opaque colony morphology. Translucent isolates (designated MO6-24/T) were also isolated. The rate of phase change was assessed by inoculating an isolated colony into L broth for overnight incubation with shaking at 30°C and then plating serial dilutions of bacteria onto L agar incubated overnight at 30°C.

Transposon mutagenesis. Tn*5* IS*50*$_L$::*phoA* (Tn*phoA*) is a Tn*5* derivative containing a gene for alkaline phosphatase lacking the promoter and signal sequences; in-frame fusion of this gene to genes encoding a secreted protein results in an active alkaline phosphatase enzyme (14). Tn*phoA* was introduced into MO6-24/O by conjugation of the vector pRT291 in *Escherichia coli* SM10 (23) with selection on L agar containing polymyxin B (50 U/ml) and kanamycin (300 μg/ml). Loss of the vector plasmid and transposition of Tn*phoA* into the chromosome was induced by the introduction of a second IncP plasmid, pHIJI, with selection on L agar containing polymyxin B (50 U/ml), kanamycin (50 μg/ml), gentamicin (30 μg/ml), glucose (0.2%), and 5-bromo-4-chloro-3-indolyl phosphate (40 μg/ml). The ratio of alkaline phosphatase-positive to alkaline phosphatase-negative transconjugants was ca. 1:200. Chromosomal DNA from 20 transconjugants, including 9 that were translucent, was examined by Southern blot analysis (13) following digestion with *Bam*HI. With the 3.4-kilobase *Hin*dIII fragment of the Tn*phoA* gene as a probe, all isolates showed a single transposon insertion.

Electron microscopy. Strains were grown on L agar for 12 h at 30°C, washed in phosphate-buffered saline, and suspended in rabbit antiserum prepared to whole cells of MO6-24/O. Preparations were incubated with frequent agi-

TABLE 1. Phase variation, hydrophobicity, and virulence of *V. vulnificus* strains[a]

Strain	Original morphology	Reversion rate[b]	Hydrophobicity[c]	50% Lethal dose (morphology[d])
MO6-24/O	O	2.2×10^{-4}	1.8	$<10^2$ (O)
MO6-24/T	T	9.2×10^{-3}	0.9	3×10^5 (O)
CVD752	T	$<10^{-5}$	0.5	3×10^6 (NR)
CVD737	T	$<10^{-5}$	0.5	1×10^7 (T)
CVD755	O	ND	1.8	$<10^2$ (ND)

[a] O, Opaque; T, translucent; ND, not done; NR, none recovered.
[b] Rate of shift to opposite morphology.
[c] Hydrophobicity, lowest molarity of $NH_4(SO_4)_2$ to cause precipitation.
[d] Morphology, colony type recovered after animal passage.

TABLE 2. Effect of sera on *V. vulnificus* strains[a]

Strain	Original morphology	Reduction in bacterial no. (log_{10})[b]	Morphology recovered
MO6-24/O	O	1.13	O
MO6-24/T	T	3.87	O
CVD737	T	>6	NG[c]
CVD752	T	>6	NG[c]
CVD755	O	0.95	O

[a] O, Opaque; T, translucent.
[b] Decrease in the number of bacteria (log_{10}) after 1 h of incubation in pooled human sera.
[c] NG, No growth.

tation for 1 h. Cells were centrifuged and prepared for electron microscopy by the method of Luft (12).

Hydrophobicity. Bacteria were grown on L agar, suspended in Tris-buffered saline which contained 0.5 M Tris (pH 7.4) and 0.8% NaCl, and standardized to an optical density at 600 nm of 1.3. Dilutions of ammonium sulfate in Tris-buffered saline ranged from 0.3 M to 4.0 M in 0.5 M increments. Cells and ammonium sulfate (25 μl each) were vortexed in microdilution dishes and incubated for 30 min at room temperature. Hydrophobicity was expressed as the molarity of the highest dilution of ammonium sulfate to cause precipitation of the bacteria.

Serum resistance. Bacteria (ca. 10^7/ml) were incubated with 65% normal, pooled human sera and 65% heat-inactivated sera at 37°C for 1 h (5); samples were incubated at 37°C to replicate in vivo temperatures. Bacteria were enumerated on L agar, and colony morphology was observed.

Utilization of transferrin-bound iron. Strains were grown overnight on L agar at 30°C and washed in phosphate-buffered saline; bacteria (ca. 10^6/ml) were inoculated into 2 ml of Chelex-deferrated synbase (Chelex 100; Bio-Rad Laboratories, Richmond, Calif.) with or without 26 μM iron-saturated transferrin (20) and incubated for 12 h at 37°C. Total viable counts were determined every 4 h on L agar, and colony morphology was observed. Transposon mutants were maintained under antibiotic selection. Utilization of transferrin-bound iron on solid medium was also assayed with L agar with the iron chelator ethylenediamine-di (*o*-hydroxyphenylacetic acid) (EDDA) (75 μg/ml) and by observing growth at 37°C of bacteria around wells containing transferrin that was 100% saturated with iron.

Animal studies. To evaluate virulence in animals, mice (n = 3 or more for each dilution) were injected intraperitoneally with 0.5 ml of serial dilutions of each strain in phosphate-buffered saline and with 0.2 ml of sterile ferric ammonium citrate (80 μg of elemental iron) in phosphate-buffered saline (25). The 50% lethal dose for each strain was calculated by the method of Reed and Muench (19).

RESULTS

Translucent variants of MO6-24/O were isolated from L broth at a frequency of 2.2×10^{-4} (Table 1). When passaged under the same in vitro conditions, translucent variants reverted back to an opaque morphology at a frequency of 9.2×10^{-3}.

Transposon mutagenesis of MO6-24/O with Tn*phoA* was confirmed by Southern blot analysis. Eight of the nine translucent transposon mutants had an insertion into what appeared to be a common chromosomal fragment. One of these eight strains was designated CVD752; the other translucent strain with a different insertion was designated CVD737. CVD752 had alkaline phosphatase activity, but CVD737 did not. These strains did not revert to the opaque phenotype when maintained under antibiotic selective pressure (Table 1). An opaque, alkaline phosphatase-positive transposon mutant was randomly selected as a positive control and was designated CVD755. Phenotypic characteristics of these transposon mutants were compared with those of the spontaneous phase variants of *V. vulnificus* MO6-24.

Encapsulation was evaluated by electron microscopy. The opaque colonial variant of MO6-24 had a ruthenium red-staining capsule (Fig. 1a), MO6-24/T had reduced amounts of capsular material (Fig. 1b), and the transposon mutants CVD752 (Fig. 1c) and CVD737 (not shown) did not appear to be encapsulated.

An increase in the degree of encapsulation for other bacterial species correlates with a decrease in hydrophobicity (1, 8, 15). We were able to detect differences in cell surface hydrophobicity by examining the relative solubility of isolates in ammonium sulfate. A decrease in capsular material as measured by the amount of ruthenium red-staining material appeared to correlate with an increase in hydrophobicity (Table 1). Correlation between translucent colony morphology and increased hydrophobicity was also observed for phase variants of six other *V. vulnificus* strains (data not shown; $P = 0.03$, Wilcoxon rank sum test, two tailed).

Both translucent transposon mutants were more sensitive to the bacterial activity of normal human sera than was the spontaneously generated MO6-24/T (Table 2). Only opaque colonies were recovered from sera that had been inoculated with MO6-24/T; no bacteria were recovered from sera inoculated with translucent transposon mutants. The opaque strain, CVD755, retained serum resistance. Heat-inactivated sera were bacteriostatic for all strains.

The change in bacterial number for the opaque and translucent variants of the parent strain and for the two transposon mutants in deferrated, defined medium with iron-saturated transferrin as the sole iron source is reported in Table 3. Both MO6-24/O and CVD737 were able to grow in this medium, which was bacteriostatic for CVD752 and bactericidal for MO6-24/T. The colony morphology for all strains was unchanged after 12 h of incubation. Defined medium without saturated transferrin was bacteriostatic for all strains. On L agar that was iron limited due to the addition of EDDA, MO6-24/O and CVD737 grew around wells containing iron-saturated transferrin after overnight incubation while no growth was observed for CVD752 and MO6-24/T after 3 days of incubation.

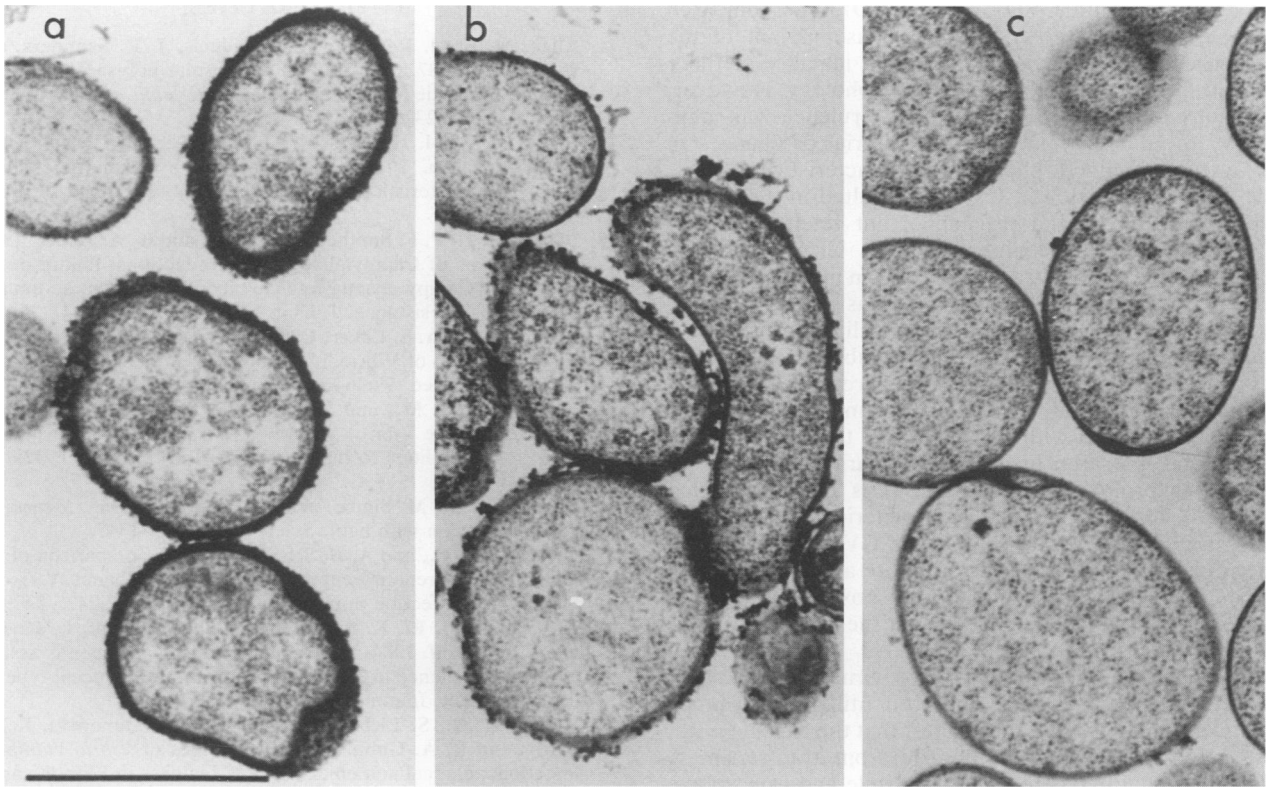

FIG. 1. Electron micrographs of MO6-24/O (a), MO6-24/T (b), and CVD737 (c) stained with ruthenium red. Bar = 1 μm.

An iron-loaded adult mouse model was used to evaluate virulence. The 50% lethal dose for both CVD737 and CVD752 was slightly higher than that for MO6-24/T (Table 1). Bacteria were recovered via cardiac puncture at 16 h postinfection from surviving animals receiving the highest inocula. Bacteria recovered from animals inoculated with MO6-24/T had an opaque morphology, while isolates from animals receiving CVD752 and CVD737 were translucent.

DISCUSSION

These data confirm previous observations regarding phenotypic characteristics of opaque and translucent strains of *V. vulnificus*. In contrast to previous reports, we were able to demonstrate reversion from a translucent to an opaque morphology. This reversibility suggests that the mechanism for phase shift does not involve deletion of genetic material (such as a prophage) or loss of a plasmid; instead, capsular expression may be controlled by a reversible genetic rearrangement that is maintained in the population. Spontaneous translucent phase variants of our parent strain reverted to an opaque morphology when grown in the presence of serum or when passaged in animals. Given the significant increase in virulence associated with an opaque, encapsulated morphology, the ability to undergo a phase shift may be important in the pathogenesis of *V. vulnificus* infections.

TABLE 3. Growth characteristics of *V. vulnificus* strains in iron-limited media (Chelex-deferrated synbase) in the presence of saturated transferrin

Strain	Change in bacterial no. (log_{10}) at:		
	4 h	8 h	12 h
MO6-24/O	−0.07	0.04	1.21
MO6-24/T	−2.42	−6.00	−6.00
CVD737	0.11	1.59	2.13
CVD752	−0.37	−0.20	−0.04

The *V. vulnificus* capsule has been defined primarily by its acidic staining properties. Attempts to associate encapsulation with differences in hydrophilicity have shown that both opaque and translucent variants of *V. vulnificus* are equally hydrophilic by hexadecane partition (21). By using ammonium sulfate precipitation, we were able to differentiate opaque and translucent variants on the basis of hydrophobicity. These data provide further evidence for the presence of a capsule and offer a method for confirmation of capsule production that is more efficient than electron microscopy.

This is the first published report of transposon mutagenesis in *V. vulnificus*. Prior to our success with pRT291, we had attempted to conjugate or transduce a number of transposon vectors, including pNK861 (24), pNK862 (24), pRT733 (23), F42 *lacI3 zzf-2*::Tn*phoA* (14), and mini-Mu Lux (6), into several *V. vulnificus* strains and were unable to demonstrate transposition (A. C. Wright, unpublished data). In this study, we identified two translucent transposon mutants that had single insertions at different sites in the chromosome. These strains did not appear to be simple phase variants; they did not revert to the opaque phenotype in vitro or in vivo, were more hydrophobic, were more serum sensitive, and had less capsule than the spontaneously generated translucent phase variant. The apparent increase in 50%

lethal dose seen with CVD752 and CVD737 as compared with that seen with the translucent phase variant of the parent strain may have been due to the inability of these mutant strains to revert to an opaque morphology. However, we cannot exclude the possibility that virulence was influenced by the decrease in capsular material or changes in other, as yet undefined, phenotypic characteristics.

The Tn*phoA* insertion in CVD737 resulted in the loss of encapsulation and serum resistance, but retention of the ability to utilize transferrin-bound iron. Growth of both CVD737 and the parent strain MO6-24/O in minimal medium with saturated transferrin was slow but was greater than that seen with CVD752 or MO6-24/T. The ability of CVD737 to utilize iron from transferrin was further substantiated by the observation that both CVD737 and the parent strain showed normal overnight growth on EDDA-containing (iron-limited) media when supplied with iron-saturated transferrin, while CVD752 and the translucent phase variant showed no growth after prolonged incubation. It is of interest that iron-limited minimal medium was bacteriocidal for MO6-24/T, while it was bacteriostatic for CVD752. Until we further define the characteristics that are affected by phase variation and the locus of the transposon mutation in CVD752, it is difficult to speculate on the reasons for this difference.

Our data indicate that at least two virulence-associated characteristics (i.e., capsule and iron utilization) can be independently inactivated and suggest that the capsule is not required for the acquisition of iron from transferrin. A similar functional independence of capsule and iron uptake has been demonstrated in *Neisseria meningitidis* (7). However, for both *N. meningitidis* (7) and *V. vulnificus*, the relevance of in vitro iron acquisition to virulence is unclear; in our studies, the ability to acquire iron from transferrin did not appear to enhance the virulence of CVD737 as compared with that of CVD752, which could not utilize transferrin-bound iron for growth in vitro. Further clarification of the role of iron acquisition mechanisms in virulence will require construction of iron transport mutants of a fully virulent strain, with efforts made to determine whether there is a decrease in virulence concomitant with the deficiency in iron transport.

In contrast to findings with CVD737, the insertional mutation in CVD752 resulted in the loss of all previously described virulence-associated characteristics. Although the exact function of the mutated gene remains to be determined, it is possible that we have interrupted a regulatory element that controls expression of multiple phenotypic characteristics. Systems of this type in which a single gene controls expression of multiple virulence-related factors have been described for a number of pathogenic bacteria (16). Our data do indicate that there is a striking loss of virulence in mice when both capsule and serum resistance are lost. While encapsulation has been shown to be the basis for serum resistance in some species (1, 3), our results are not sufficient to demonstrate a causal relationship between these characteristics. Further analysis of mutant strains should help to define the relationship of capsule and other virulence-associated characteristics to the pathogenesis of *V. vulnificus* infections and to determine if the capsule is the primary factor affecting virulence.

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