

Correlation between Virulence and Colony Morphology in *Vibrio vulnificus*

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Of 38 isolates of *Vibrio vulnificus* examined, all avirulent strains produced only translucent colonies. All virulent strains, with the exception of biogroup 2 (eel pathogens), exhibited both opaque and translucent colonies. Isogenic morphotypes were examined for a variety of phenotypic and virulence traits. Only the ability to utilize transferrin-bound iron and the presence of a surface polysaccharide were found to correlate with colony opacity and virulence.

Vibrio vulnificus is an estuarine bacterium capable of producing both wound infections and septicemia in humans (19). Infection generally develops following ingestion of the organism (typically by consumption of raw oysters) (3) or exposure of a skin break to seawater containing the bacterium (8). Persons with elevated serum iron levels are significantly predisposed to such infection; in these cases the mortality rate may exceed 60% (3, 19). A variety of potential virulence factors have been examined in both clinical and environmental strains (15). In 1981, Kreger et al. (10) presented data suggesting that the virulence of *V. vulnificus* may be attributed, at least in part, to the presence of an antiphagocytic surface antigen. Kreger and co-workers later showed that this antigen is an acidic polysaccharide and that antiserum to this fraction would protect mice against injections of live cells of *V. vulnificus* (11). An avirulent strain (A1402) was seen to lack significant amounts of this surface polysaccharide. Independently, Amako et al. (1) reported that a clinical isolate of *V. vulnificus* had a heavy "capsule" of ruthenium red-staining material, while strain ATCC 27562 contained a mixture of both encapsulated and nonencapsulated cells. These researchers were able to correlate the proportion of stained cells of the two strains with virulence in mice and with serum sensitivity. More recently, Yoshida et al. (24) reported variations in colony opacity within single strains of *V. vulnificus*. They found that serum resistance, antiphagocytic activity, tissue invasiveness, and lethality were correlated with colony opacity, as well as with the presence of capsular material. We have noted previously the rather common presence of colony variants in numerous isolates of *V. vulnificus* obtained from both clinical and environmental sources. Some strains have even been received from major culture collections with such notations as "... some original cultures showed 2 colony types. . . ." Based on the results of these and other studies noting differences in such traits as flagellation (13), serology (17), lactose fermentation (1), outer membrane protein pattern (23), siderophore production (18, 23), and enzyme production (15), we decided to investigate the nature and significance of the isogenic variants and to determine whether they are common to all strains of *V. vulnificus*.

We examined 38 strains of *V. vulnificus* including both clinical and environmental isolates and virulent and avirulent

strains. When cultures were plated onto a variety of media (including thiosulfate-citrate-bile salts-sucrose, heart infusion, brain heart infusion, and lactose agar), we observed either mixtures of opaque and translucent colonies or translucent colonies only for all 38 strains. Mixed-colony-type cultures appeared to arise independently of the plating medium employed, although we found that unsupplemented heart infusion agar (Difco Laboratories; 1.5% agar added) provided an excellent contrast for the two colony types. When an opaque colony was selected from one of the mixed cultures and replated, the rate of colony dissociation to the translucent type was found to vary from less than 1 to ca. 7%. Although several media and hundreds of plates were examined, reversion of translucent colonies to the opaque type was never observed. This is in contrast to the observations of Yoshida et al. (24), who reported reversion of both colony types at rates of ca. 10^{-4} . We saw the highest rates of opaque-to-translucent variation with cells taken from a maintenance medium (Bacto-Peptone, 10 g; cysteine hydrochloride, 20 mg; agar, 6 g; NaCl, 23.4 g, $MgSO_3 \cdot 7H_2O$, 7.0 g; KCl, 0.75 g; distilled water, 1 liter), in which over 60% of the colonies were frequently observed to be translucent (Fig. 1).

To confirm that both types were in fact *V. vulnificus*, hybridization studies were performed by using a cytolysin-cytotoxin gene probe which is specific for *V. vulnificus* (21; J. G. Morris, Jr., A. C. Wright, D. M. Roberts, P. K. Wood, L. M. Simpson, and J. D. Oliver, Appl. Environ. Microbiol., in press). Both colony types were hemolytic and hybridized with the probe. Given the importance of lactose fermentation in the taxonomy of *V. vulnificus*, along with reports associating this fermentation with the development of mutant cells (2), it seemed possible that the development of the translucent cell type might correspond to these lactose-fermenting mutants. Neither colony type was able to rapidly ferment lactose when inoculated into the BM-lactose medium described by Baumann et al. (2).

A 50% lethal dose was determined with the isogenic variants of one strain (C7184), with values of 4×10^6 and 1.9×10^8 being observed for the opaque and translucent types, respectively. Using an adult mouse model (14), we examined the 38 strains of *V. vulnificus* for virulence and colony opacity. With the exception of biogroup 2 (20), we found a 100% correlation between virulence and colony morphology (Table 1), with the cultures composed solely of translucent colonies being avirulent (inocula of 10^9 did not produce

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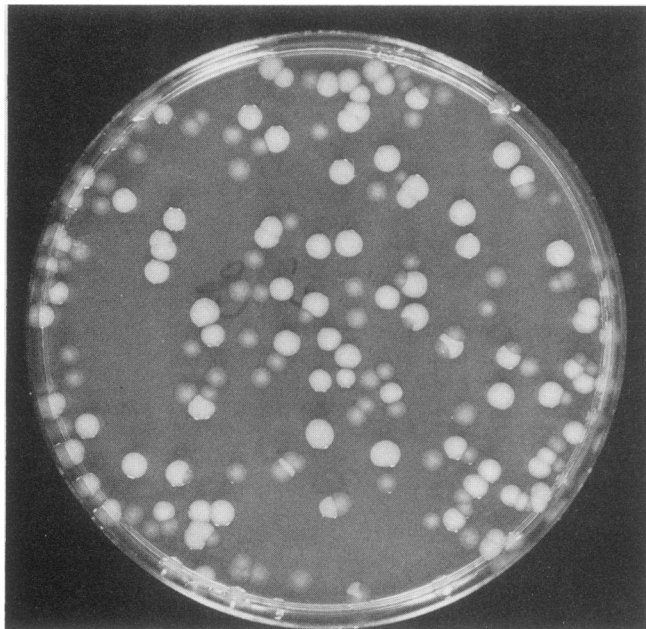


FIG. 1. Opaque and translucent colonies of *V. vulnificus* C7184.

fatality in mice). The two strains of the eel pathogen (*V. vulnificus* biogroup 2), although translucent, were also virulent for adult mice. The reason that biogroup 2 of *V. vulnificus* differs from biogroup 1 is not known, but biogroup 2 may possess virulence traits different from those of human-pathogenic biogroup 1 since no studies of its virulence factors have been reported.

To confirm that the differences in lethality were associated with the presence of an external ruthenium red-staining layer, we examined the isogenic variants of strain C7184 by electron microscopy after staining for acidic polysaccharides as described by Luft (12). While some cells from opaque colonies lacked appreciable amounts of this material, the great majority possessed a significant layer of ruthenium red-staining material (Fig. 2A). In contrast, cells from translucent colonies lacked this external layer (Fig. 2B). Thus, as reported by Amako et al. (1), Kreger et al. (10), and Yoshida et al. (24), virulence appears to correlate with the presence of this external layer.

Additional testing of the two morphotypes was undertaken to identify differences which might contribute to virulence. Outer membrane proteins were prepared from both cell types of C7184 by two different methods (5, 8), electrophoresed, and detected with both Coomassie blue and a commercial silver stain kit (Bio-Rad Laboratories). No outer membrane protein differences could be detected between either cell type. When the surface hydrophobicity of the two cell types was examined by using the hexadecane partitioning method described by Parker and Munn (16), no differences were observed (both cell types were highly hydrophilic). Because motility has also been reported to be involved in virulence in some bacteria and because Maeda et al. (13) had reported differences in flagellation between two strains of *V. vulnificus*, we used the flagellum-staining method of Heimbrook et al. (Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, R22, p. 240) to determine whether such variation could result from opaque and translucent variants within a strain. Cells taken directly from both opaque and translucent colonies revealed only single polar flagella. Plasmid extraction

was attempted by using the methods described by Davidson and Oliver (6); no plasmids were detected in either cell type.

We have recently reported that although the strains of *V. vulnificus* tested were unable to obtain iron from transferrin when it was only 30% iron saturated, some strains could utilize transferrin-bound iron at 100% saturation (L. M. Simpson and J. D. Oliver, submitted for publication). Moreover, we have recently found a correlation between the ability to utilize transferrin-bound iron and virulence (J. G. Morris, Jr., A. C. Wright, L. M. Simpson, P. K. Wood, D. E. Johnson, and J. D. Oliver, submitted for publication). In light of our observations regarding the two morphotypes exhibited by *V. vulnificus*, we reexamined iron utilization in the two morphotypes of strain C7184. We found that only the opaque cells were able to utilize transferrin-bound iron. In examining the response of isogenic variants to iron overload in an adult mouse model (22), we found that the 50% lethal dose for the translucent variant (unlike the opaque strain) was not lowered with injections of iron. In the remaining 37 strains of *V. vulnificus*, we found that the opaque strains were virulent in the iron overload model (inoculum, 10^3), although none of the translucent strains was lethal at that level (Table 1).

Colony dissociation has been described for numerous bacterial species, including marine bacteria (7). In an early review of the literature, Braun (4) concluded that colony dissociation resulted from the development of mutants which became established in the culture. If we are observing such a mutational event in *V. vulnificus*, then it is evident that our culture conditions strongly select for the translucent population. Alternatively, the conditions of our cell culture (or maintenance) may be enhancing a specific genetic alteration in the opaque cell type. In either case, it appears that the genetic event is highly unidirectional, since we observed only opaque-to-translucent dissociation and not the reverse. We are currently investigating several aspects of the molecular genetics of both colony morphotypes.

One of the important consequences of our findings regarding the absolute correlation between colony opacity and virulence is the possibility that during the performance of lethality studies a translucent colony may be inadvertently selected. This may be a particular problem when thiosulfate-citrate-bile salts-sucrose agar is used to monitor culture purity, since we saw only small differences in colony morphology on this medium. If a translucent colony is selected, then a significantly increased 50% lethal dose may result. This is a consequence not only of the inherently lower virulence of this cell type but also of the lack of reversion to the more virulent opaque type. For example, *V. vulnificus* E4125 was reported to be avirulent (A. L. Reyes, J. T. Peeler, C. H. Johnson, P. L. Spaulding, and G. N. Stelma, Jr., Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, P2, p. 275), yet we found this strain to be lethal for mice. Furthermore, we found their strain to be translucent upon receipt,

TABLE 1. Virulence and colony opacity of *V. vulnificus*

Colony type	No. of strains	Virulence ^a	Iron responsive ^b
Opaque	26	+	+
Translucent	10	-	-
Translucent (biotype 2)	2	+	NT

^a Lethal for adult mice at an inoculum of 10^9 (see reference 14 for a description of the adult mouse model).

^b Lethal for adult mice at an inoculum of 10^3 when concurrently injected with 534 μ g of ferric ammonium citrate (80 μ g of Fe^{3+}). NT, Not tested.

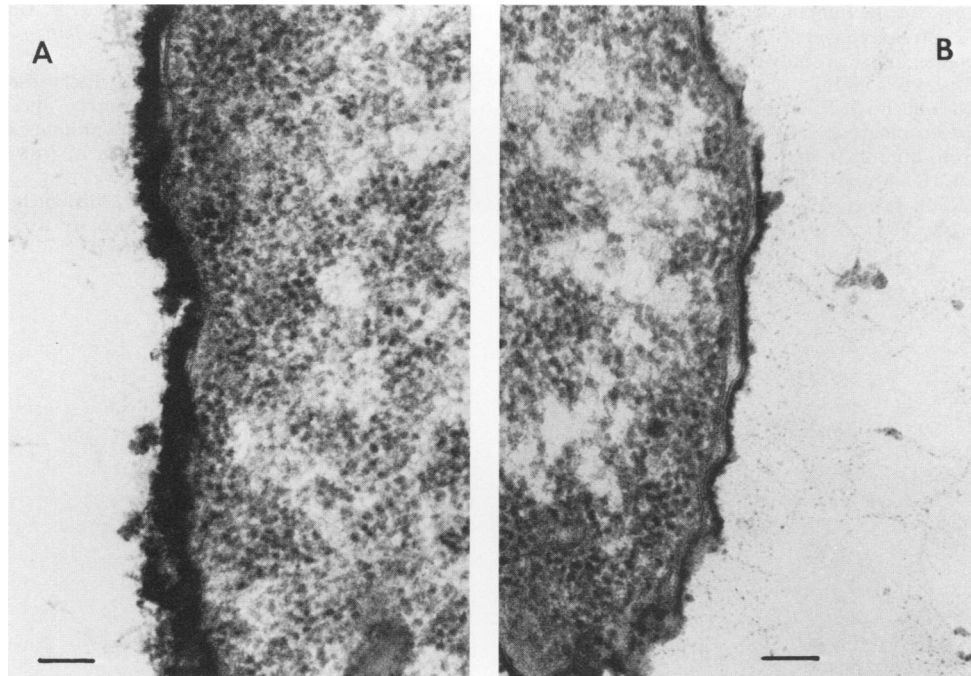


FIG. 2. Electron micrograph of cells from opaque and translucent colonies of *V. vulnificus* C7184. Cells were stained for acidic polysaccharide by using ruthenium red as described by Luft (12). (A) Opaque cell type, showing thick polysaccharide layer outside the cell envelope. (B) Translucent cell type, demonstrating thin layer of ruthenium red-staining polysaccharide. Bars, 0.1 μm .

whereas our own culture was of the opaque type. We have found that similar inconsistencies in the results of virulence studies performed in other laboratories were due to this colony morphology variation.

It is not known whether both of the two cell types exist in nature, and if they do, the percentage of the *V. vulnificus* population each type represents. It is conceivable that a significant percentage of naturally occurring cells of *V. vulnificus* may exist in the translucent phase. In this case, the lower virulence of this cell type may help to explain, along with the apparent requirement for host iron overload, the relatively small number of cases of infection reported with this human pathogen.

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