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Expression of *Vibrio vulnificus* Capsular Polysaccharide Inhibits Biofilm Formation

Lavin A. Joseph and Anita C. Wright*

Aquatic Food Products Laboratory, Food Science and Human Nutrition Department, University of Florida, Gainesville, Florida 32611

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***Vibrio vulnificus* is a human pathogen that produces lethal septicemia in susceptible persons, and the primary virulence factor for this organism is capsular polysaccharide (CPS). The role of the capsule in *V. vulnificus* biofilms was examined under a variety of conditions, by using either defined CPS mutants or spontaneous CPS expression phase variants derived from multiple strains. CPS expression was shown to inhibit attachment and biofilm formation, which contrasted with other studies describing polysaccharides as integral to biofilms in related species.**

Vibrio vulnificus is indigenous to estuarine environments (9, 18, 24, 36, 43) and causes human infections associated with raw oyster consumption (3). Pathogenesis was recently reviewed, and virulence is primarily attributed to capsular polysaccharide (CPS) expression (34). Opaque (O) colony morphology, indicative of a virulent, encapsulated phenotype, exhibits reversible phase variation to translucent (T) colony types with reduced CPS expression and decreased virulence (30, 47). Defined mutations in the CPS operon confirmed the relationship of CPS and virulence (28, 42, 44, 45). *Vibrio* spp. attach to algae and zooplankton (5, 15, 16, 17, 19, 22), and *V. vulnificus* may be more concentrated in oysters and fish which feed on these organisms (9, 29, 36, 43). Microbial communities attached to nutrient-rich surfaces are generally referred to as biofilms and are thought to engage in complex signaling for expression of CPS and other factors (7, 25, 26). For example, *V. cholerae* biofilms require production of polysaccharide, pili, and flagella (5, 21, 23, 37, 38, 39, 46). Biofilms for *V. vulnificus* biotype 2 eel pathogens were recently described (20); however, this group differs from human pathogens of biotype 1 in that biotype 2 lipopolysaccharide (LPS) is homogeneous (serovar E) and CPS may not always be required for virulence (2). The role of CPS in biofilms of either biotype has not been addressed; therefore, our studies examined *V. vulnificus* biofilms in O versus T phase variants and CPS mutants that differed in their abilities to produce capsular polysaccharide.

CPS expression inhibits *V. vulnificus* biofilm formation. Surface CPS displays a continuum of expression among *V. vulnificus* strains (44). Strains for this study are detailed in Table 1 and were stored at −70°C in 50% glycerol to ensure stability of phase variants. O strains are completely encapsulated, while T strains either are acapsular or have reduced, patchy capsules. Mutant strains are acapsular but differ in CPS biosynthesis: CVD752 contains a polar transposon mutation in the CPS operon that eliminates biosynthesis, while MO6-24/31T contains a nonpolar mutation, specifically targeting the CPS transport function of the *wza* gene, and can synthesize CPS but is unable to transport it to the cell surface (44, 45).

Biofilm formation on abiotic surfaces was examined by crystal violet absorption assays, and the relative biofilm content was estimated from the concentration of dye eluted from destained cells and matrix (32). Examination of staining capacity for dilutions of suspended cultures indicated slightly higher absorption (less than twofold) for O or T strains than for mutants, but eluted dye reflected a linear relationship to cell density independent of surface properties for all strains (not shown). Biofilm formation was initially examined with log-phase cultures (1 ml) incubated for 6 h statically in glass tubes with Luria-Bertani broth (LB) at 30°C. Attached cells were washed in phosphate-buffered saline (PBS), stained with crystal violet (1%), rinsed, and destained with acetic acid (33%). The optical density of eluted dye was measured at 570 nm (Molecular Devices). Encapsulated M06-24/O showed a more-than-threefold decrease in attached cells compared to partially encapsulated M06-24/T (Fig. 1), suggesting that CPS expression inhibits biofilm formation. However, the phase variation mutation(s) is not defined and may be pleiotropic; therefore, the inhibitory function of CPS was confirmed by observation of increased biofilms for both defined CPS mutants compared to MO6-24/O. We note that bacteria may produce multiple types of CPS, and a role for other polysaccharides in *V. vulnificus* biofilms is still a possibility.

Influence of growth conditions. Environmental conditions influence biofilm formation in *V. cholerae* (1, 15, 16, 19, 21) and CPS expression in *V. vulnificus* (44); therefore, biofilms were examined for cultures grown statically in LB at different pHs (6, 7, and 8), temperatures (25, 30, and 37°C), and salinities (1 and 2% NaCl). Log-phase cultures were inoculated into fresh media (10^6 CFU in 100 μl) and monitored over 48 h in a microtiter assay (Immulon 1B; Dynex) by methanol fixation, crystal violet staining, and acetic acid elution (32). Duplicate independent experiments with triplicate samples were performed for each condition. Room temperature and pH 6 produced minimal biofilm for all strains independent of other factors (not shown). Encapsulated MO6-24/O exhibited only minimal biofilm under any condition, and significant differences ($P < 0.05$) between O and either T or mutant strains

* Corresponding author. Mailing address: Food Science and Human Nutrition Department, Bldg. 475, Newell Dr., P.O. Box 110370, University of Florida, Gainesville, FL 32611. Phone: (352) 392-1991. Fax: (352) 392-9467. E-mail: acwright@ifas.ufl.edu.

TABLE 1. *V. vulnificus* strains and CPS expression

Strain	Description (reference)
MO6-24/O	Opaque clinical isolate; expresses CPS over entire cell surface (44)
MO6-24/T	Translucent phase variant of MO6-24/O, reduced patchy capsule (44)
MO6-24/31T	Acapsular MO6-24/O mutant; does not transport CPS to the cell surface due to mutation in CPS transport gene, *wza* (45)
CVD752	Acapsular MO6-24/O mutant; does not produce CPS due to polar transposon insertion (45)
LC4/O	Clinical isolate; expresses CPS over the entire cell surface (unpublished)
LC4/T	Translucent phase variant of LC4/O; acapsular (unpublished)
C7184/O	Clinical isolate; expresses CPS on the entire cell surface (30)
C7184/T	Translucent phase variant of C7184/T; acapsular (30)

were observed at several time points (Fig. 2). Differences could not be attributed to growth kinetics, as strains have similar growth rates (44), and optical densities (A_{600}) of cultures did not correlate with attached biofilms. The greatest biofilm was observed at 1% NaCl for pH 7 or 8 and 30 or 37°C, in contrast with *V. cholerae* biofilm formation, which was optimum at pH 2 and increased with temperature (15). However, both species generally exhibit greater attachment at 1% than at 2% salinity. CPS inhibition of biofilms was also observed for O versus T variants of other strains (LC4 and C7184), but conditions producing optimum biofilm formation varied among strains (not shown).

Listeria monocytogenes biofilm formation was greater on hydrophobic polyvinyl chloride than on more-hydrophilic stainless steel surfaces (10), and increased cell surface hydrophobicity may promote biofilms (31). *V. vulnificus* CPS expression greatly decreases cell surface hydrophobicity (42), presumably because hydrophilic CPS masks more-hydrophobic structures, such as pili. Therefore, the contribution of substrate properties to attachment was examined by using hydrophobic (Immulon 1B) versus more hydrophilic (Immulon 2 or glass) surfaces, but no significant differences were observed (not shown). Thus, although more hydrophobic acapsular strains have increased adherence to surfaces, it would be misleading to conclude that attachment was due solely to hydrophobic interactions.

Biofilms and nutrient status. Biofilm formation may be a response to nutrient limitation, with biofilms initially forming at nutrient-rich surfaces and then detaching as nutrient availability declines following extended incubation (26). For example, starvation of a marine vibrio increased adhesion to glass surfaces (8). As shown in Fig. 3, nutrient-depleted (48 h of preincubation in PBS) *V. vulnificus* MO6-24/T ($P < 0.05$) and mutants (not significant) showed increased biofilm formation in comparison to nonstarved cells. Also, the *V. vulnificus* biofilm generally accumulated over 24 h and then leveled off or declined by 48 h, suggesting that nutrient-limited cells were detaching (Fig. 2).

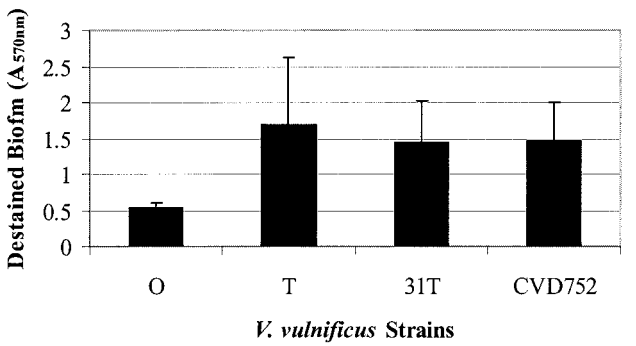

FIG. 1. Biofilm formation of *V. vulnificus* on glass. Strains of *V. vulnificus*, including MO6-24/O (O), MO6-24/T (T), MO6-24/31T (31T), and CVD752, were incubated in LB in glass tubes for 6 h at 30°C and stained with crystal violet (32). The optical density at 570 nm of eluted dye from attached cells is indicative of relative bacterial cell concentration in biofilms.

Starvation may also increase phase variation. Extracellular polysaccharide (EPS) is required for *V. cholerae* biofilm and shows phase variation whereby rugose (wrinkled) colonies express EPS and smooth colonies do not. Starved *V. cholerae* demonstrates increased phase shift to rugose, biofilm-forming variants (21). However, comparable increases in *V. vulnificus* phase shift as a function of starvation or extended incubation were not observed, and strains maintained the original phenotype. Thus, the *V. vulnificus* starvation response was independent of phase variation. Increased rugose-colony phase shift in response to specific growth medium, through induction of high-frequency phase variation, was also reported (1). Perhaps more extended incubation or as yet unidentified nutrient parameters may influence *V. vulnificus* phase variation and biofilm formation.

Biofilm structure. Fluorescence microscopy (HB-10101A; Nikon; DC290 camera; Eastman Kodak), using BacLight viability staining (Molecular Probes), confirmed strain differences in biofilm formation. After 24 h at 30°C in polystyrene plates (Corning), cells were rinsed twice with PBS and stained. Attached MO6-24/O cells appeared mostly as singles or doublets, while dense biofilms through multiple focal planes were observed for acapsular strains (Fig. 4). Interestingly, all attached O cells were viable (as indicated by yellow-green fluorescence), while biofilms of other strains consisted of both live and dead (red) cells. Encapsulated cells did not form the monolayers seen with EPS-negative, biofilm-defective *V. cholerae* (38, 46) or *Escherichia coli* (6); thus, *V. vulnificus* CPS probably relates more to initial attachment rather than the later stages of biofilm development postulated for the role of other polysaccharides.

Conclusions. Polysaccharides are not always critical to initial adhesion but are considered major constituents of the complex architecture of later stages of biofilm formation (6, 12, 38). Surface polysaccharides include CPS, EPS (slime), and LPS, but distinctions are not clear. For example, bacteria may produce multiple types of CPS or have LPS capsules (*E. coli* K_{LPS}) comprised of CPS sugars attached to lipid A (40). Polysaccharides, derived from the same genetic locus, are referred to as EPSs in mucoid strains and CPS in nonmucoid isolates; how-

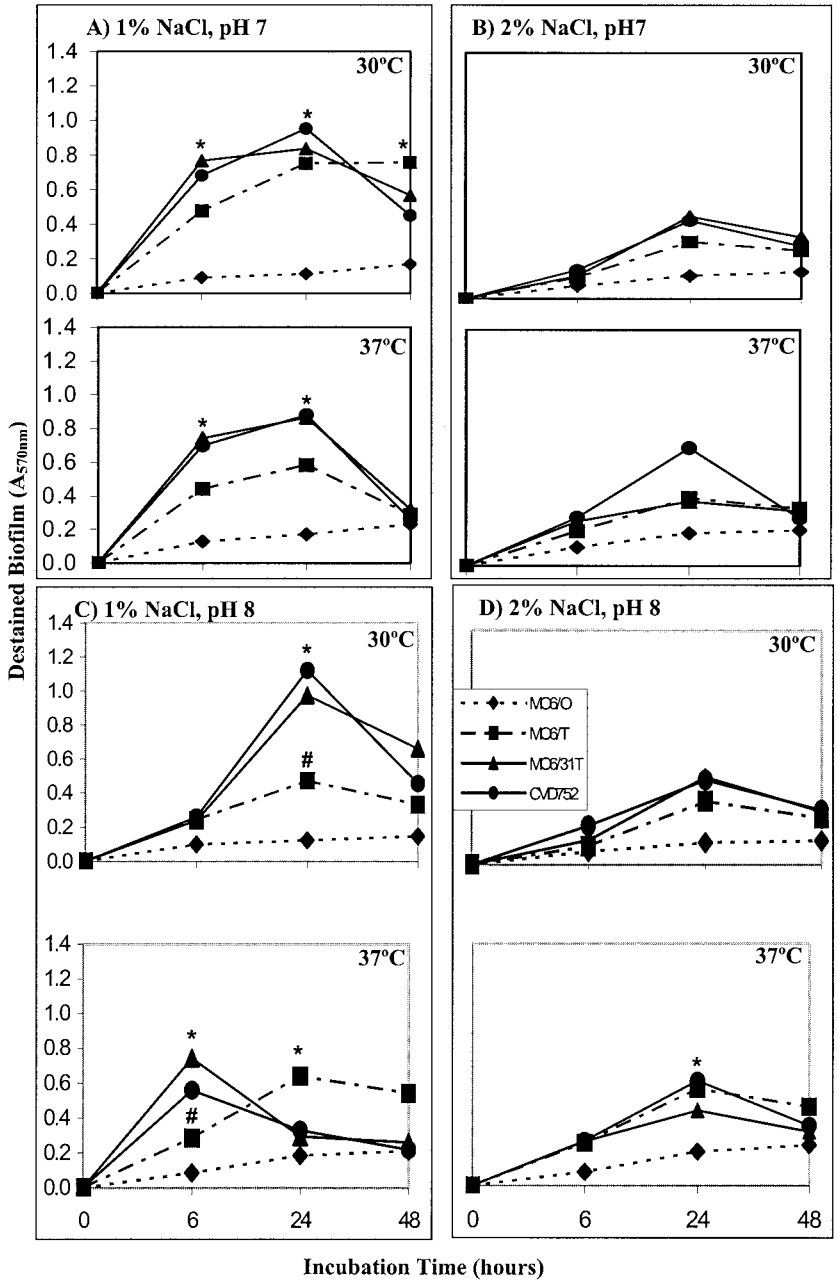

FIG. 2. Biofilm formation for *V. vulnificus* strains under different growth conditions. Strains of *V. vulnificus*, including MO6-24/O, MO6-24/T, MO6-24/31T, and CVD752, were examined for biofilm formation in microtiter plates in LB prepared with either 1% NaCl, pH 7 (A), 2% NaCl, pH 7 (B), 1% NaCl, pH 8 (C), or 2% NaCl, pH 8 (D) at incubation temperatures of 30 and 37°C as indicated. The optical density at 540 nm of eluted dye from attached cells is indicative of relative bacterial cell concentration in biofilms. Significant differences ($P < 0.05$) in destained biofilms between MO6-24/O and other strains (*) and between MO6-24/T and acapsular mutants (#) are noted.

ever, EPS-producing *V. cholerae* strains are not mucoid but instead exhibit rugose morphology. Further, EPS has also been referred to as an extracellular polymeric substance (41), and polysaccharide may or may not be a component of this matrix.

Our results indicated that *V. vulnificus* CPS expression actually inhibited attachment and biofilm formation, and similar observations were reported elsewhere (D. Ramos, K. Piechaczek, and P. Watnick, Abstr. 103rd Gen. Meet. Am. Soc. Microbiol, abstr. J-022, p. 358, 2003). Thus, contrasting roles for *V. vulnificus* and *V. cholerae* polysaccharides are proposed and may be related to their divergent biochemical properties. Uronic acid sugars, common to *V. vulnificus* CPS (4, 14), contribute to increased negative charge and hydrophilicity (42), while *V. cholerae* EPS is composed primarily of neutral sugars glucose and galactose (46). Unlike *V. vulnificus*, EPS-producing cells are strongly adherent to each other as well as surfaces. Further, oral biofilms are also composed primarily of neutral sugars (35). Uronic acid sugars from mucoid *E. coli* (6) and

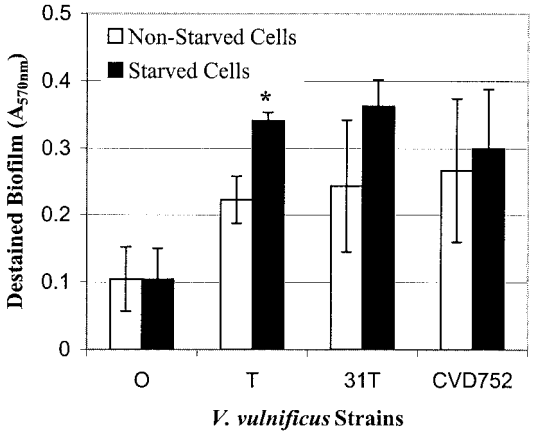

FIG. 3. Biofilm formation of starved versus nonstarved *V. vulnificus* strains. Strains of *V. vulnificus* (MO6-24/O, MO6-24/T, MO6-24/31T, and CVD752) were assayed for biofilms with or without prior starvation (48 h in PBS) in LB for 6 h at 30°C. The optical density at 540 nm of eluted dye from attached cells is indicative of relative bacterial cell concentration in biofilms. Significant differences ($P < 0.05$) in biofilm formation between starved and nonstarved cells of the same strain are noted (*).

Pseudomonas spp. (11) were previously implicated in biofilm formation; however, recent analysis indicated that glucose, not alginate, predominates in the *Pseudomonas aeruginosa* EPS (41). This study demonstrated that an alginate-negative *algD* mutant formed a biofilm equivalent to those formed by encapsulated, nonmucoid wild-type strains and questioned the role of uronic acid in biofilm formation in nonmucoid strains. These data underscore the importance of CPS composition and indicate that polysaccharide function may relate to both structure and relative quantity of capsule expressed.

Our studies suggest that environmental conditions can decrease biofilm. In light of the purported contributions of biofilms to survival (7, 8, 21, 23, 26, 27, 31, 33, 46), manipulating *V. vulnificus* biofilms could be used to reduce seafood contamination. Increased biofilm capacity of T variants compared to that of O variants might predict the prevalence of T variants in estuarine environments; however, environmental isolates are almost always opaque and presumably encapsulated (43). Additional factors, such as avoidance of phagocytic cells, may provide increased selection for encapsulated variants in mol-

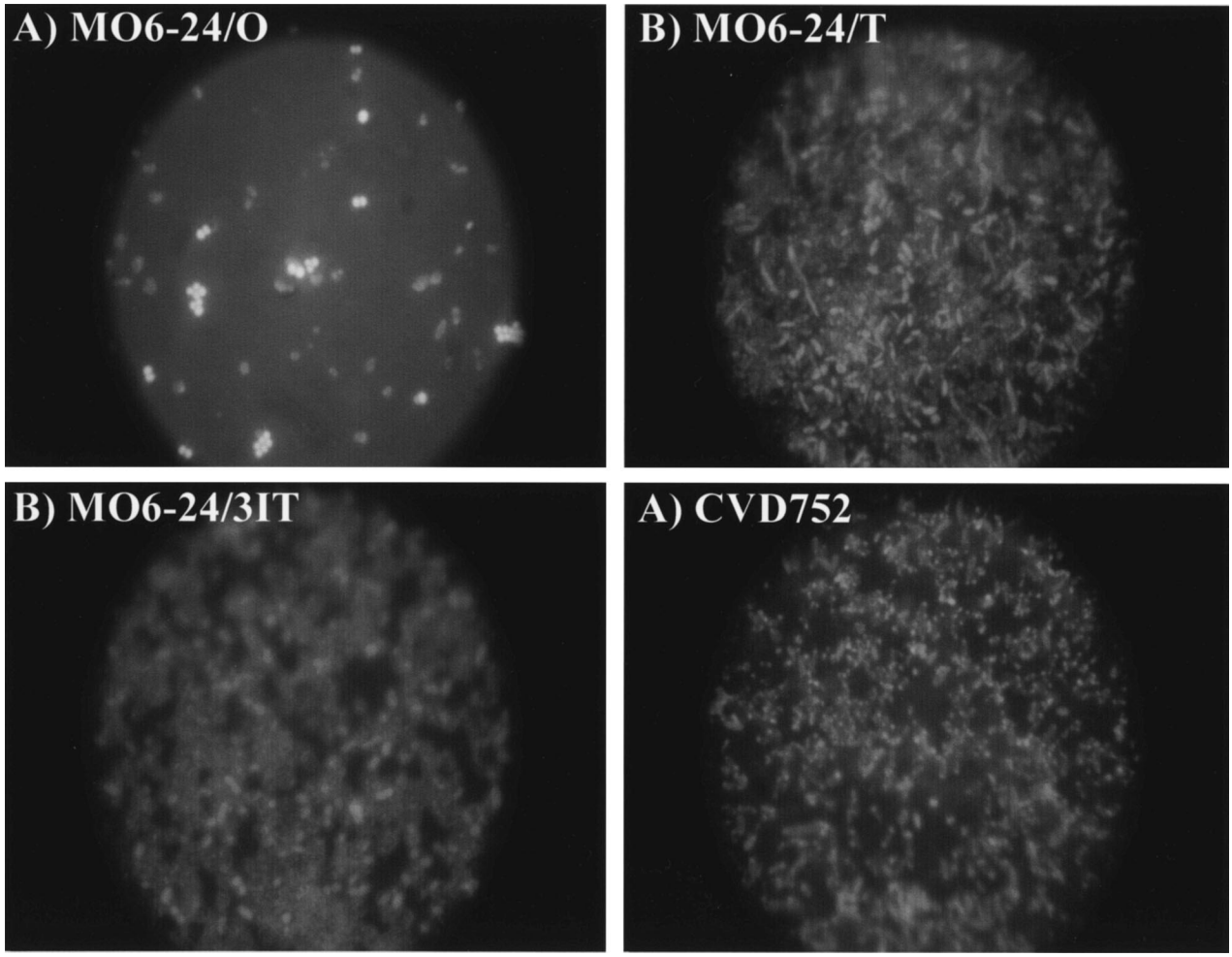

FIG. 4. Biofilm formation of *V. vulnificus* strains as observed with fluorescence microscopy. Strains of *V. vulnificus* (MO6-24/O [A], MO6-24/T [B], MO6-24/31T [C], and CVD752 [D]) were observed for biofilm formation after 6 h of incubation in LB at 30°C by fluorescence microscopy as described in the text. BacLight staining provides discrimination between live and dead cells (green versus red; color not shown). MO6-24/O cells were observed as viable, while the other strains were a mixture of live and dead cells.

luscan hosts (13). Alternatively, attachment to surfaces may vary with the biological context. For example, encapsulated *Klebsiella pneumoniae* was less adherent than acapsular mutants to most tissue culture cell lines but attached well to mucus-producing cells (12). Eel mucous also increased the adhesion of *V. vulnificus* biotype 2 (2). Sutherland (35) emphasized that assumptions about biofilm are frequently based on structures derived from monocultures and polysaccharides extracted from planktonic cells; therefore, further examination of the relationship of polysaccharide structure, biochemistry, and genetics to natural biofilms is needed to delineate the complex parameters influencing these microbial communities.

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