

csrA Inhibits the Formation of Biofilms by *Vibrio vulnificus*[∇]

Melissa K. Jones, Elizabeth B. Warner, and James D. Oliver*

Department of Biology, University of North Carolina at Charlotte, Charlotte, North Carolina 28223

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PCR screening of the shellfish-borne pathogen *Vibrio vulnificus* revealed *csrA*-negative strains, and these strains formed increased biofilm compared to *csrA*-positive strains. Complementation in *trans* with *csrA* resulted in reduced biofilm formation, similar to that by *csrA*⁺ strains. Our results provide evidence that *csrA* inhibits biofilm formation in *V. vulnificus*.

The opportunistic human pathogen *Vibrio vulnificus* is an inhabitant of marine environments worldwide (2, 6, 12, 14, 20). It is responsible for 95% of all seafood-related fatalities in the United States, with its most serious disease (primary septicemia) associated with the consumption of raw or undercooked oysters (15). Clinical and environmental strains of this bacterium can be sorted through rapid PCR screening of a gene (*vcg*) that has been correlated with virulence in this pathogen. Strains which possess the sequence associated with clinical isolates (*vcgC*) are designated C type, while strains containing the sequence associated almost exclusively with environmental isolates (*vcgE*) are designated E type (17). Both of these genotypes have been isolated from the environment, and yet their distribution among environmental sources varies. For example, we found E-type strains to be overwhelmingly predominant in oysters compared to C-type strains, indicating a differential ability for strains with this genotype to colonize oyster tissue (19). Attachment to oysters likely involves the formation of biofilms, and several surface structures have been shown to be involved in biofilm formation by this organism. However, of these structures, only capsular polysaccharide (CPS) has also been shown to be differentially expressed between the C and E genotypes. The absence of surface capsule has been linked to increased biofilm formation in *V. vulnificus*, and E-type strains demonstrate an increased transition to a phenotype with reduced capsule (CPS) expression (5, 8).

Given these indications that E-type isolates may form increased biofilm compared with C-type isolates, the two genotypes were compared in their abilities to form biofilms by using a microtiter plate method. Briefly, overnight cultures of *V. vulnificus* were inoculated at a 1:100 dilution into 99 μ l of Luria broth (LB) contained in 96-well microtiter plates (Costar). Cells were grown statically at 30°C for 48 h. Following incubation, the wells were emptied and then washed three times with 150 μ l of one-half-strength artificial seawater. Two hundred microliters of 1% crystal violet was added to the washed wells and incubated at room temperature for 15 min. The crystal violet was removed, and the wells were washed three times with 200 μ l of one-half-strength artificial seawater. The

remaining dye was solubilized in 200 μ l of 95% ethanol, and the absorbance values were measured using a uQuant Microplate spectrophotometer (Biotek). Results from this assay showed that upon screening of 18 C-type and 16 E-type strains, there was no significant difference in the level of biofilm formation between the genotypes (data not shown). However, when these strains were grouped by isolation source, oyster isolates demonstrated a significantly ($P = 0.021$) higher level of biofilm formation than did strains from all other sources of isolation (Fig. 1).

The global regulatory protein CsrA has been shown to influence biofilm formation by *Escherichia coli* by binding to mRNA and affecting translation (7, 11). Microarray analysis comparing clinical and environmental strains of *V. vulnificus* found *csrA* to be missing in many environmental isolates (G. Vora, personal communication). Therefore, strains previously analyzed for biofilm formation were screened for the presence of *csrA* using PCR. BLAST comparison of *E. coli* K-12 CsrA (accession no. AAC75738) with the two published *V. vulnificus* genomes (CMCP6, accession no. NC_004459, and YJ016, accession no. NC_005139) was used to locate *csrA* in *V. vulnificus*, and results showed 96% amino acid identity and 78% nucleotide identity between *E. coli* and both *V. vulnificus* strains. Alignment of the *V. vulnificus* nucleotide sequences (YJ016 [accession no. VV2801] and CMCP6 [accession no. VV1_1595]) revealed 100% nucleotide identity for *csrA* and 99% nucleotide identity for the surrounding regions (1 kb upstream and downstream).

Two sets of primers were developed for PCR screening. The first primer set localized to a conserved region within *csrA* (*csrA*_{up}, 5'-GCGTAGGCGAAACACTGA-3', and *csrA*_{down}, 5'-CGTTGCCTTTCTCAGCC-3'), while the second pair of primers were found in conserved regions upstream and downstream of the gene (*csrA*_{AF2}, 5'-GTCAGCCTCTATCATTCA GAG-3', and *csrA*_{AR1}, 5'-GGATAATAGCCTCGTAGCTA-3', respectively). Cycling conditions for both primer pairs were optimized using a gradient thermal cycler (Techne), and the following parameters were used for amplification: 94°C for 3 min followed by 30 cycles of 94°C for 45 s, 58°C for 45 s, and 72°C for 45 s with a final extension at 72°C for 2 min. All strains were initially screened with *csrA*_{up} and *csrA*_{down}. Strains yielding a negative PCR result were then screened with the second *csrA* primer set and with primers for *vvhA* (19) to verify that the isolates were *V. vulnificus* and the DNA was amplifiable. Strains which yielded negative results for both *csrA* primer sets

* Corresponding author. Mailing address: Department of Biology, University of North Carolina at Charlotte, Charlotte, NC 28223. Phone: (704) 687-8516. Fax: (704) 687-3457. E-mail: jdoliver@uncc.edu.

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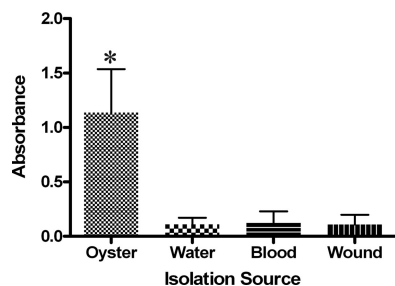


FIG. 1. Biofilm formation by *V. vulnificus* isolates based on their source of isolation. Those strains ($n = 9$) isolated from oysters demonstrated a significantly (asterisk; $P = 0.021$) higher level of biofilm formation than did strains isolated from other sources (nine water, nine blood, and nine wound isolates).

and a positive result with *vhA* primers were designated *csrA* negative. Results showed that only 33.3% of the oyster isolates previously examined for biofilm formation were positive for *csrA* (henceforth designated *csrA*⁺), while 66.7% of water isolates and 100% of both blood and wound isolates were *csrA*⁺. These data suggested that *csrA* may be absent with greater frequency in oyster isolates than in strains from other environmental sources. However, a more extensive screening of additional strains revealed that 72.3% of oyster isolates ($n = 47$), 72.7% of water isolates ($n = 32$), and 100% of blood and wound isolates ($n = 18$ and 9, respectively) were positive for *csrA*, indicating that there is no correlation between isolation source and the presence of *csrA*.

The oyster isolates which formed high levels of biofilm were predominately *csrA* negative, suggesting that *csrA* may be involved in biofilm formation by *V. vulnificus*. To investigate this hypothesis, biofilm formation by *csrA*⁺ ($n = 17$) and that by *csrA*-negative ($n = 12$) strains isolated from both clinical and environmental sources were compared. Results showed that strains without *csrA* produced significantly ($P \leq 0.001$) more biofilm than did strains possessing the gene, indicating that the presence of *csrA* negatively influences biofilm formation by *V. vulnificus* (Fig. 2). To confirm this hypothesis, a *csrA*-negative strain was complemented in *trans* with *csrA*. Primers *csrAF2* and *csrAR1* (located 28 bp upstream and 49 bp downstream of *csrA*, respectively) were used to amplify the gene in strain *V. vulnificus* C7184/K2 by using the PCR conditions described above, and the resulting product was cloned into the TOPO TA cloning vector as described by the manufacturer (Invitrogen).

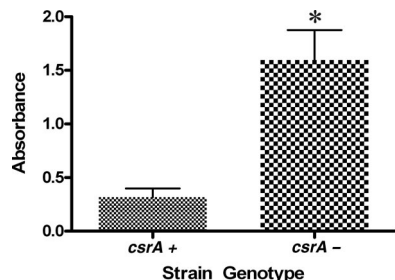


FIG. 2. Biofilm formation abilities of *csrA*⁺ ($n = 17$) and *csrA*-negative ($n = 12$) strains. Strains which lacked *csrA* (*csrA*⁻) displayed significantly (asterisk; $P \leq 0.001$) higher biofilm formation than did strains which possessed *csrA*.

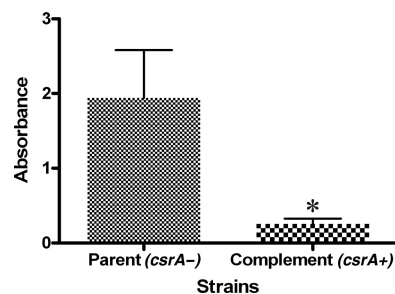


FIG. 3. Complementation in *trans* with pRKCSRA resulted in a significant (asterisk; $P \leq 0.005$) reduction in biofilm formation compared to that by the *csrA*-negative (*csrA*⁻) parent strain.

The presence of *csrA* in the resulting construct (pTCSRA) was confirmed by PCR using *csrA*_{up} and *csrA*_{dn} primers (data not shown). pTCSRA and the broad-host-range vector pRK404 were digested with EcoRI per the manufacturer's instructions (Promega), and the digests were gel purified and cleaned using the Ultra-Clean Gel Spin kit (Mo-Bio). Digested pRK404 and *csrA* were then ligated overnight at 4°C using T4 DNA ligase per the manufacturer's instructions (Promega) to yield pRKCSRA, which was then cloned into chemically competent *E. coli* S17-λ-pir. pRKCSRA was conjugated into the *csrA*-negative *V. vulnificus* strain NRO 102-418 through filter mating as previously described (21). Complementation was confirmed by PCR using *csrA*_{up} and *csrA*_{dn}, and the complement was then evaluated for biofilm formation. Results showed that complementation in *trans* of *csrA* significantly ($P \leq 0.005$) reduced biofilm formation compared to the parent strain, and levels were similar to those of *csrA*⁺ isolates (Fig. 3). These results strongly suggest that *csrA* is an inhibitor of biofilm formation by *V. vulnificus*.

V. vulnificus is a natural inhabitant of oysters, and many studies have investigated physical properties which contribute to its attachment to and colonization of such shellfish. Several surface structures (including CPS, extracellular polysaccharide, pili, and flagellum) are involved in biofilm formation by this organism (3, 4, 8, 9, 16). However, the genetic regulators responsible for their expression during biofilm formation have yet to be elucidated. Recently, the global carbon storage regulator CsrA has been identified as a major posttranscriptional regulator of biofilm formation by *E. coli* (7). This protein often binds to mRNA transcripts and promotes their decay, thereby impacting gene expression at the translational level (1).

In this study biofilm formation by isolates of *V. vulnificus* was examined, and results indicated that strains lacking *csrA* formed significantly ($P \leq 0.001$) higher levels of biofilm than did *csrA*⁺ strains, indicating that *csrA* is an inhibitor of biofilm formation by this species. This observation was confirmed through complementation of a *csrA*-negative strain, which resulted in a significant ($P \leq 0.005$) reduction in biofilm formation compared to that by the parent strain. In *E. coli*, biofilm formation is inhibited by CsrA through downregulation of both glycogen synthesis and production of a polysaccharide adhesion which is required for biofilm formation to occur (7, 18). Conversely, in *V. vulnificus* CPS expression has been reported to reduce biofilm formation (8). Our studies demonstrated that *csrA* negatively regulates biofilm formation (as is seen in *E.*

coli), suggesting that either (i) *csrA* is not a regulator of group I polysaccharide synthesis or (ii) there are other factors in addition to the presence of CPS required for biofilm formation. Recently, however, several studies have reported that capsule expression does not play a role in biofilm formation by *V. vulnificus*, throwing the role of CPS in biofilm formation into question (3, 10, 13). Together, these findings suggest that *csrA* may be an important regulator in colonization by *V. vulnificus* and highlight the need for more investigation into the role of this protein in polysaccharide production and biofilm formation.

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