CHARACTERIZATION OF THE TYPE IV PILI OF *VIBRIO PARAHAEMOLYTICUS*

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INTRODUCTION

Bacteria that belong to the family *Vibrionaceae* are ubiquitous in the marine environment, especially in coastal waters. These bacteria accumulate in shellfish and are responsible for the majority of seafood-associated infections in the U.S. primarily through consumption of raw or improperly cooked shellfish, mostly oysters. *Vibrio parahaemolyticus* and *Vibrio vulnificus* are responsible for over 50% of the *Vibrio* infections reported in the U.S. We are characterizing a number of bacterial surface proteins to determine their function in persistence of *V. parahaemolyticus* and *V. vulnificus* in the marine environment and in oysters, as well as their role in mammalian pathogenesis. Our initial work has focused on Type IV pili (TFP), thin hair-like structures that have been shown to be important in the attachment of many Gram-negative pathogens to a variety of surfaces. Molecular characterization and comparisons of the genome sequences of several *Vibrio* species has shown that all express more than one Type IV pilus. Our studies on one of these, the *pilA*-encoded TFP *in V. vulnificus*, have demonstrated that this TFP contributes to adherence to human epithelial cells, biofilm formation, virulence, and persistence in oysters. The roles of homologous TFPs of *V. parahaemolyticus,* PilA and MshA, in adherence to epithelial cells, biofilm formation, and persistence in oysters will be discussed.

BACKGROUND

Vibrios persist in a wide range of temperatures and salinities, associate with aquatic flora, and concentrate in shellfish such as oysters through filter-feeding (11). In the U.S., the majority of seafood-related, bacterial infections in humans are due to two members of the species, *Vibrio vulnificus* and *Vibrio parahaemolyticus*. *V. vulnificus*, is commonly found in tropical or sub-tropical waters, and is capable of causing severe illness and death in susceptible individuals (4, 16). *V. parahaemolyticus*, however, proliferates in temperate as well as sub-tropical and tropical environments. This bacterium can cause severe gastroenteritis in healthy individuals, and is responsible for several seafood-related outbreaks of illness including the recent outbreaks in 1997 and 2006 in the Pacific Northwest, 1998 in Texas and New England and 2004 in Alaska. These cases were all traced to the consumption of raw oysters (1, 7, 9).

Vibrios concentrate in shellfish such as oysters through filter feeding and their presence in shellfish and in the environment is not correlated with anthropogenic pollutants such as fecal coliforms. In the warmer summer months, concentrations of vibrios in oysters have

been reported to be 100-fold higher than that in the surrounding water (17). Post harvest methods typically used to reduce concentrations of these bacteria in shellfish such as relaying and depuration are effective is eliminating indicator bacteria such as fecal coliforms, but have been inconsistent in reducing concentrations of vibrios (12). Differences in post-harvest handling and storage conditions can also influence the survival and growth of vibrios in oyster tissue allowing these bacteria to multiply several-fold during this period (5). Since current processing and handling techniques appear to be inadequate to reduce the number of vibrios from oysters, a better understanding of the mechanisms by which these bacteria remain associated with shellfish is necessary to develop effective methods for their reduction or elimination after harvest.

The ability of bacteria to adhere to a variety of surfaces is an essential step for their survival in various niches, and is mediated by several mechanisms. One mechanism of adherence is by the interaction of pili, thin, long structures protruding from the surface of bacteria that are involved in adhesion to both biotic and abiotic surfaces. One type of pili, the type IV pili commonly found in several pathogens, are involved in adherence and colonization of mammalian epithelial cells have been implicated as a virulence factor because of their role in adherence and colonization of mammalian epithelial cells(6). In *V. cholerae*, the TFP, MSHA, has a role in attachment to zooplankton and may therefore be important for persistence in the environment (3). A second TFP, PilA is upregulated by exposure of the bacterium to chitin (10) and appears to facilitate the ability of *V. cholerae* to take up exogenous DNA thereby providing a mechanism for increasing genetic diversity. *V. cholerae* also expresses a third TFP, the toxin co-regulated pilus or TCP that has a primary role in colonization and virulence, but is also associated with survival of the bacterium in the marine environment (15).

Our previous work has shown that PilA TFP of *V. vulnificus* is involved in adherence to human epithelial cells as well as biofilm formation on abiotic surfaces. PilA is also necessary for virulence in a mouse model as well as in colonization of oysters, suggesting that this pilin has a role both in mammalian pathogenesis as well as in persistence in the environment (14; unpublished work). The *V. vulnificus* genome also encodes a second TFP, MSHA, but only a single type IV prepilin peptidase gene, *pilD* (2), which encodes an enzyme essential assembly of pilin subunits into pili. In our earlier studies, absence of *pilD* resulted in loss of all surface pili and a significantly greater defect in adherence to human epithelial cells as well as persistence in oysters (13), suggesting that both pilins likely have a role in colonization of oysters. In this study we examine the role of homologous TFP of *V. parahaemolyticus* in adherence to epithelial cells, biofilm formation, and persistence in oysters.

METHODS

Construction of V. parahaemolyticus *strains with mutations in* pilA *and* mshA.

The gene *V10* from *V. parahaemolyticus* is homologous to *mshA* of *V. cholerae* and *V. vulnificus*. Genetic and transcriptional analysis of *V. cholerae mshA* shows that it is part of a cluster of 16 genes transcriptionally organized into two operons: a secretory operon encoding nine genes and a structural operon with seven genes (Figure 1A). The *mshA* gene is part of the structural operon and encodes the structural pilin subunit, with the

other genes in the operon encoding minor pilins as well as inner and outer membrane proteins that may be required for pilus assembly. A non-polar mutation was constructed in *V10 (mshA)* from *V. parahaemolyticus*, using a system that utilizes crossover PCR to create in-frame, deletions in chromosomal DNA (8). The recombinants were selected by antibiotic resistance and confirmed by Southern blot hybridization.

The *pilA* gene from *V. parahaemolyticus* is clustered with three other pilus biogenesis genes *(pilABCD)*, homologous to *V. vulnificus* and *V. cholerae* (Figure 1B). A non-polar deletion mutation was similarly constructed in this gene using the same strategy as for *mshA*, with allelic exchange confirmed by Southern blot hybridization. A *V. parahaemolyticus* strain with mutations in both *pilA* and *V10* (*mshA*) was also constructed.

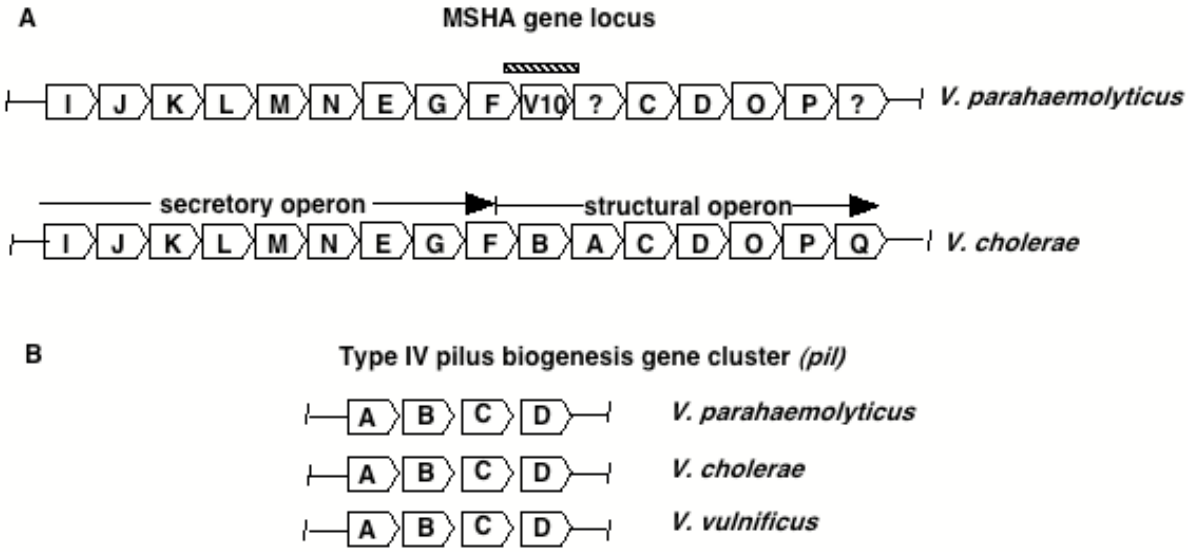

***Figure 1. A) Schematic representation of the mannose-sensitive hemagglutinin (*msh*) gene locus of* V. cholerae *and the homologous locus of* V. parahaemolyticus*. The locus in the former contains a secretory operon and a structural operon as indicated. B) Schematic representation of the type IV pilus biogenesis genes (pil) of* V. parahaemolytics *and the homologous clusters in* V. cholerae *and* V. vulnificus**

Complementation of the V10 (mshA) *and* pilA *mutants.* To confirm that the *V10* and *pilA* mutations can be restored functionally and that the deletions in these genes do not affect the function of other genes in the cluster, the genes *V10* and *pilA* were cloned in a plasmid with an inducible *tac* promoter. The plasmids were then conjugated into the strains with the respective pilin mutations.

RESULTS

Comparison of the wild type and genetically inactivated strains for phenotypic differences.

Biofilm formation: Biofilm formation was compared on borosilicate glass following previously published protocols (14). Cultures of the wild-type, *pilA* mutant, *mshA* mutant, the double mutant (*pilA;mshA*) and the complemented mutant strains were grown in Marine broth (Difco) and TCG broth at 25°C. There was a significant defect in biofilm formation by strains with mutations in both *pilA* and *mshA*, and this defect was restored in the complemented mutant strains.

Adherence to human epithelial cells: Quantitative adherence assays were performed on human epithelial cell (HeLa) following previously published protocols (14). In these assays there was a slight but measurable defect in adherence of the *pilA* mutant strain as compared to the wild-type strain. However adherence of the *mshA* mutant strain was not significantly different than that of the wild-type strain, suggesting that the *mshA* pilin is not required for adherence to human epithelial cells.

DISCUSSION

This study suggests that the *pilA*-encoded pilus of *V. parahaemolyticus* contributes to biofilm formation on abiotic surfaces as well as adherence to human epithelial cells. The role of both the pilins, PilA and MshA in adherence to oysters as well as in virulence are currently being investigated. The clarification of the roles of both type IV pili as specific adherence and colonization factors may be useful in targeting these adhesions in the development of methods to specifically purge oysters of these bacteria as well as for therapeutic intervention in human infections.

LITERATURE CITED

1. *Vibrio parahaemolyticus* Infections Associated with Consumption of Raw Shellfish --- Three States, 2006. MMWR. 55.
2. Chen, C. Y., K. M. Wu, Y. C. Chang, C. H. Chang, H. C. Tsai, T. L. Liao, Y. M. Liu, H. J. Chen, A. B. Shen, J. C. Li, T. L. Su, C. P. Shao, C. T. Lee, L. I. Hor, and S. F. Tsai. 2003. Comparative genome analysis of *Vibrio vulnificus*, a marine pathogen. Genome Res 13**:**2577-87.
3. Chiavelli, D. A., J. W. Marsh, and R. K. Taylor**.** 2001. The mannose-sensitive hemagglutinin of *Vibrio cholerae* promotes adherence to zooplankton. Appl Environ Microbiol 67**:**3220-5.
4. Colwell, R. R. 1996. Global climate and infectious disease: the cholera paradigm. Science 274:2025-31.
5. Cook, D. W**.** 1994. Effect of time and temperature on multiplication of *Vibrio vulnificus* in postharvest Gulf Coast shellstock oysters. Appl Environ Microbiol 60**:**3483-4.
6. Craig, L., M. E. Pique, and J. A. Tainer. 2004. Type IV pilus structure and bacterial pathogenicity. Nat Rev Microbiol **2:**363-78.
7. DePaola, A., C. A. Kaysner, J. Bowers, and D. W. Cook**.** 2000. Environmental investigations of *Vibrio parahaemolyticus* in oysters after outbreaks in Washington, Texas, and New York (1997 and 1998). Appl Environ Microbiol 66**:**4649-54.

8. Link, A. J., D. Phillips, and G. M. Church. 1997. Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. J Bacteriol 179**:**6228-37.
9. McLaughlin, J. B., A. DePaola, C. A. Bopp, K. A. Martinek, N. P. Napolilli, C. G. Allison, S. L. Murray, E. C. Thompson, M. M. Bird, and J. P. Middaugh. 2005. Outbreak of *Vibrio parahaemolyticus* gastroenteritis associated with Alaskan oysters. N Engl J Med 353:1463-70.
10. Meibom, K. L., X. B. Li, A. T. Nielsen, C. Y. Wu, S. Roseman, and G. K. Schoolnik. 2004. The *Vibrio cholerae* chitin utilization program. Proc Natl Acad Sci U S A 101**:**2524-9.
11. Morris, J. G., Jr. 2003. Cholera and other types of vibriosis: a story of human pandemics and oysters on the half shell. Clin Infect Dis 37**:**272-80.
12. Oliver, J. D., and J. B. Kaper**.** 1997. Food Microbiology- Fundamental and Frontiers. ASM Press. Washington D. C.
13. Paranjpye, R. N., J. C. Lara, J. C. Pepe, C. M. Pepe, and M. S. Strom. 1998. The type IV leader peptidase/*N*-methyltransferase of *Vibrio vulnificus* controls factors required for adherence to HEp-2 cells and virulence in iron-overloaded mice. Infect Immun 66**:**5659-68.
14. Paranjpye, R. N., and M. S. Strom. 2005. A *Vibrio vulnificus* type IV pilin contributes to biofilm formation, adherence to epithelial cells, and virulence. Infect Immun 73:1411-22.
15. Reguera, G., and R. Kolter. 2005. Virulence and the environment: a novel role for *Vibrio cholerae* toxin-coregulated pili in biofilm formation on chitin. J Bacteriol 187**:**3551-5.
16. Strom, M. S., and R. N. Paranjpye**.** 2000. Epidemiology and pathogenesis of *Vibrio vulnificus*. Microbes Infect 2**:**177-88.
17. Wright, A. C., R. T. Hill, J. A. Johnson, M. C. Roghman, R. R. Colwell, and J. G. Morris, Jr**.** 1996. Distribution of *Vibrio vulnificus* in the Chesapeake Bay. Appl Environ Microbiol 62**:**717-24.

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