

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

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Vibrio vulnificus expresses a number of potential virulence determinants that may contribute to its ability to cause a severe and rapidly disseminating septicemia in susceptible hosts. We have cloned and characterized two genes encoding products related to components of the type IV pilus biogenesis and general secretory (type II) pathways by complementation of a type IV peptidase/*N*-methyltransferase (PilD) mutant of *Pseudomonas aeruginosa* with a *V. vulnificus* genomic library. One of the genes (*vvpD*) encodes a protein homologous to PilD and other members of the type IV peptidase family that completely restores this activity in a *P. aeruginosa* mutant deficient in the expression of PilD. The other gene (*vvpC*) encodes a homolog of PilC from *P. aeruginosa*, where it is essential for assembly of type IV pili. Phenotypic characterization of a *V. vulnificus vvpD* mutant, constructed by allelic exchange, showed that VvpD is required for the expression of surface pili, suggesting that the pili observed on *V. vulnificus* are of the type IV class. This mutant was also unable to secrete at least three extracellular degradative enzymes, and the localization of one of these (the cytolysin/hemolysin) to the periplasmic space indicates that these proteins are normally exported via the type II secretion pathway. Loss of VvpD resulted in significant decreases in CHO cell cytotoxicity, adherence to HEp-2 cells, and virulence in a mouse model. Capsule formation and serum resistance were not affected in the *vvpD* mutant, indicating that in addition to capsule, virulence of *V. vulnificus* requires type IV pili and/or extracellular secretion of several exoenzymes.

Vibrio vulnificus biotype 1 is an estuarine bacterium that can cause primary septicemia as well as serious wound infections (34, 35). While septicemia occurs primarily in immunocompromised individuals or those that suffer from cirrhosis or hemochromatosis, healthy people can become infected through wounds. Septicemia can develop after ingestion of shellfish carrying the organism, with the greatest risk coming from the consumption of raw oysters (5, 21). Mortality in these cases exceeds 50%, increasing to greater than 90% in people who go into shock or become hypotensive shortly after admission to a hospital (22). As many as 50% of all vibrio-related illnesses in the United States are caused by *V. vulnificus* (7). Recently, a second biotype of *V. vulnificus*, biotype 2, has been implicated in septicemic infections of cultured eels (63). Animal studies have shown that biotype 2 is also capable of causing septicemia in mammals, including opportunistic infections of humans (3, 4).

Among the many factors implicated as possible virulence determinants for *V. vulnificus* are extracellular toxins and enzymes (e.g., cytolysin and elastolytic protease) (25, 33), a polysaccharide capsule (67), resistance to phagocytosis (19, 70), resistance to the bactericidal effects of human sera (19, 67, 70), and the ability to acquire iron from transferrin (51). *V. vulnificus* undergoes a phase variation between virulent and avirulent forms, with the former being encapsulated and serum resistant and the latter having lost these traits (49, 67). In animal models, encapsulation is clearly an important determinant of virulence (67, 70), most likely because the capsule confers

serum resistance and is antiphagocytic (49). The role of the cytolysin is less clear, as cytolysin-negative strains have the same 50% lethal dose (LD₅₀) for mice as wild-type strains (64).

Type IV pili have been shown to be important adherence factors in many gram-negative bacteria (57). Biogenesis of these pili is in part controlled by the type IV leader peptidase, a bifunctional enzyme that proteolytically cleaves the specialized leader sequence of type IV pilin precursors followed by *N*-methylation of the newly exposed N-terminal amino acid before assembly into the pilus structure (38, 60). Additional proteins required for type IV pilus biogenesis also have this specialized leader sequence (1, 31), and though it has yet to be demonstrated directly, it is presumed that these pilin-like proteins are substrates of the type IV peptidase as well. In addition to pilus biogenesis, the type IV peptidase is required for extracellular secretion of proteins via the general secretory (type II secretion) pathway (GSP) (43, 45). At least four GSP-associated proteins with the type IV leader sequence are processed by this peptidase (37, 44, 60), and as demonstrated directly in *Pseudomonas aeruginosa* (58), *Aeromonas hydrophila* (40), and more recently *Legionella pneumophila* (9a, 27) type IV leader peptidase mutants are unable to secrete proteins via the GSP.

We have cloned and sequenced two genes from *V. vulnificus* that encode homologs of components of the type IV pilus biogenesis and type II secretion pathways. One of these, *vvpC*, encodes a polypeptide that is highly homologous to PilC, a protein of unknown function that is required for assembly of type IV pili in *P. aeruginosa* (36). The other, *vvpD*, encodes a homolog of the bifunctional type IV leader peptidase/*N*-methyltransferases found in many bacterial genera (28). We con-

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TABLE 1. Bacterial strains, plasmids and phage used

Strain, plasmid, or phage	Relevant characteristics	Source or reference(s)
<i>V. vulnificus</i>		
MO6-24/OP	Wild type (clinical isolate)	J. D. Oliver
C7184/OP	Wild type (clinical isolate)	J. D. Oliver
PAC1	Wild type (environmental isolate)	M. Coyle
C7184-D ₁₂ Ω	C7184 with disrupted <i>vvpD</i>	This study
C7184-402K	C7184 with disrupted <i>vwhA</i>	This laboratory
<i>E. coli</i>		
DH5α	<i>supE44 lacU169(φlacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Bethesda Research Laboratories
S17-λpir	<i>supE44 hsdR endA1 recA thi pro RP4-2-Tc::Mu-kan::Tn7 (λpir)</i>	32, 50
<i>P. aeruginosa</i>		
PAK	Wild type	S. Lory
PAK 2B18	<i>pilD::Tn5</i>	S. Lory (36)
PAK-CΩ	PAK mutant with the Ω interposon inserted in the <i>pilC</i> gene	23
Cloning vectors		
pLAFR3	Tc ^r broad-host-range plasmid vector	52
pBluescript II SK ⁺	Ap ^r phagemid cloning vector	Stratagene
pMMB67HE.cam	Cm ^r Ap ^r broad-host-range cloning vector, <i>lacI^q/tac</i> promoter	17
pEP185.2	Cm ^r suicide vector	20
pZErO-2	Kan ^r Zero background cloning vector	Invitrogen
Recombinant plasmids		
pJCL12B	25-kb <i>Sau3AI</i> fragment from <i>V. vulnificus</i> MO6-24 in pLAFR3	This study
pMS450	4.8-kb <i>PstI</i> fragment from pJCL12B cloned into pLAFR3 (<i>vvpBCD</i>)	This study
pMS451	2.8-kb <i>HindIII-PstI</i> fragment cloned into pLAFR3 (<i>vvpCD</i>)	This study
pMS4514	2.8-kb <i>HindIII-PstI</i> fragment cloned into pBluescript II SK ⁺	This study
pMS451Ω	2.8-kb <i>HindIII-PstI</i> fragment with Ω fragment inserted in the <i>NarI</i> site of <i>vvpD</i>	This study
pMS451Ω-2	4.8-kb <i>KpnI-SmaI</i> fragment from pMS451Ω in pEP185.2	This study
pRPD1	1.0-kb <i>Sall</i> fragment from C7184 cloned into pMMB67HE.cam (<i>vvpD</i>)	This study
Bacteriophage PO4	<i>P. aeruginosa</i> pilus-specific phage	S. Lory

structed a mutant unable to express VvpD and examined effects of the mutation with respect to expression of pili, extracellular protein secretion, capsule expression, tissue culture cytotoxicity and adherence, and virulence. We show that in the absence of VvpD, the mutant is significantly reduced in all of these functions except capsule formation. This is the first demonstration of a *V. vulnificus* mutation affecting expression of factors other than capsule that results in decreased virulence of the organism.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Unless noted, *V. vulnificus* strains were grown at 30°C in brain heart infusion (BHI) broth, while *Escherichia coli* and *P. aeruginosa* strains were grown at 37°C in Luria-Bertani (LB) broth. Antibiotic concentrations (micrograms per milliliter) used were as follows: chloramphenicol, 30; streptomycin, 25; spectinomycin, 25; and tetracycline, 20. Polymyxin B was used at 50 U/ml, and isopropylthio-β-D-galactoside (IPTG) was used at a final concentration of 1 mM.

DNA manipulations. *V. vulnificus* chromosomal DNA was extracted following procedures described for *P. aeruginosa* (56). Routine plasmid and cosmid DNA extractions were performed by the alkaline lysis method (6), while Qiagen midiprep columns (Qiagen, Santa Clarita, Calif.) were used for large-scale plasmid preparations. Standard techniques were used for enzymatic manipulations, ligations, transformations, and DNA electrophoresis (47). Probes used for Southern blot analyses were prepared by the random priming method incorporating digoxigenin-labeled dUTP, followed by chemiluminescence detection with anti-digoxigenin-alkaline phosphatase conjugate and disodium 3-(4-methoxyphospho) [1,2-dioxetane-3,2'-(5-chloro)tricyclo[3.3.1.1^{3,7}]decan-4-yl] phenyl phosphate (Genius system nonradioactive detection kit, version 2.0; Boehringer Mannheim, Indianapolis, Ind.).

Construction of a *V. vulnificus* genomic library. A *V. vulnificus* genomic library was constructed by ligation of chromosomal DNA partially digested with *Sau3AI* from strain MO6-24 into the cosmid vector pLAFR3 (52) as described by Pepe et al. (40).

Nucleotide sequence determination. Double-stranded DNA sequencing of pMS450, containing a 4.8-kb *PstI* fragment from *V. vulnificus* MO6-24, was

performed by the dideoxy-chain termination method (48). Primers were synthesized with a Gene Assembler Special (Pharmacia, Piscataway, N.J.) or purchased commercially. Subsequent homology searches were performed by using BLAST (basic local alignment search tool) (2).

The *vvpC* and *vvpD* genes of strain C7184 were amplified by PCR using *V. vulnificus* C7184 chromosomal DNA as the template and the following primers: 5'-CTATCAATGGAAAGGGATTACGG-3' and 5'-AGCCAAACAGGGAAGCT-3' to generate a 1,308-bp fragment containing *vvpC*; and 5'-GCCGGATGCCAATGTA-3' and 5'-GACGATATCGATGGCAA-3' to generate an overlapping 1,319-bp fragment encompassing *vvpD*. The fragments were purified on Nucleospin columns (Machery-Nagel GmbH & Co., Düren, Germany), and both strands were sequenced by the ABI Prism 310 Dye Terminator Cycle sequencing protocol (Applied Biosystems Inc., Perkin-Elmer, Foster City, Calif.).

Construction of a VvpD overexpression plasmid. A plasmid construct in which *vvpD* is located downstream of an inducible *tac* promoter was constructed by the following method. A primer pair was designed to amplify the *V. vulnificus vvpD* gene with a *HindIII* site (underlined) and a ribosome binding site (boldface) immediately upstream of the initiating methionine codon (italicized) of *vvpD* (5'-CACCACAAAGCTTAAAGGAGATTATATATATGGAC-3') and a *Sall* site (underlined) (3'-TGTCTGCGTTCGACGATATCGATGGCAA-5') 92 bp downstream of the termination codon. Using these primers, a 1-kb fragment containing the entire *vvpD* gene was amplified from *V. vulnificus* C7184 by PCR, digested with *HindIII* and *Sall*, subcloned into pBluescript II SK⁺, and subsequently subcloned into pMMB67HE.cam immediately downstream of the *tac* promoter to generate pRPD1. pRPD1 was then conjugated into C7184D₁₂Ω by triparental matings (10) with *E. coli* DH5α(pRK2013) for complementation experiments.

Prepilin peptidase assay. Strains C7184 and C7184D₁₂Ω carrying the vector pMMB67HE.cam and strain C7184D₁₂Ω carrying pRPD1 were grown overnight at 30°C in BHI broth with chloramphenicol (2.5 μg/ml) and IPTG added to a final concentration of 1 mM. Preparation of membrane fractions and subsequent prepilin leader peptidase assays using purified prepilin from *P. aeruginosa* as the substrate were performed as previously described (59). Samples were run on a sodium dodecyl sulfate-Tricine-15% polyacrylamide gel and stained with Coomassie blue R-250.

Cell fractionation and cytolysin assay. Overnight cultures of strains to be tested were incubated overnight at 37°C at 200 rpm in BHI broth with or without chloramphenicol (2.5 μg/ml), subcultured into BHI broth or BHI broth plus chloramphenicol, grown to late log phase (optical density at 600 nm of 2.0), and then centrifuged to separate the cells from the supernatant fraction. The pelleted cells were fractionated to recover the periplasmic contents by osmotic shock (69),

and the cytoplasmic fractions were isolated as described by Manoil and Beckwith (30). Cytolysin activity was measured in the supernatant, periplasmic, and cytoplasmic fractions in 96-well microtiter plates. Serial twofold dilutions of 0.1-ml samples in phosphate-buffered saline, pH 7.4 (PBS), were incubated with an equal volume of a 0.8% suspension of sheep erythrocytes for 2 h at 37°C (15). Hemolytic indices were determined as the reciprocal of the last dilution in which complete lysis of erythrocytes was observed.

Other enzyme assays. Chitinase activity was determined by the conventional plate method (39). Plates were spot inoculated on nutrient agar containing 0.5% NaCl and 5 µg of chloramphenicol per ml, overlaid with 10 ml of the same agar containing a 1% (wt/vol) colloidal chitin suspension, and incubated at 25°C for 7 days. A narrow (1- to 2-mm) zone of clearing around bacterial growth was indicative of chitinase secretion. Protease secretion was similarly determined on BHI agar plates containing 1% skim milk incubated at 30°C overnight (26). Secretion was verified by a 2- to 3-mm zone of clearing around the area of growth of the streaked culture.

Phage PO4 sensitivity assay. *P. aeruginosa* strains to be tested for the presence of pili were grown overnight at 37°C on LB agar plates with the appropriate antibiotics. Cells were suspended in LB broth and then streaked onto LB agar plates. One microliter of the pilus-specific phage PO4 (~10¹⁰ PFU) was spotted onto the center of each streak, and plates were incubated at 37°C. Sensitivity was defined as the absence of growth in the area of phage inoculation.

Serum sensitivity assay. A 0.5-ml aliquot of saline-washed bacterial cells containing 10⁷ to 10⁸ CFU ml⁻¹ was mixed with 0.5 ml of fresh human serum or heat-inactivated serum (56°C, 30 min) and incubated at 37°C for 1 h. The number of bacteria in serial 10-fold dilutions before and after incubation was counted on LB agar after overnight incubation at 37°C. Serum resistance was measured as the log of the ratio of the initial concentration of bacteria to the number recovered after 1 h.

TEM. To visualize pili by transmission electron microscopy (TEM), bacterial cells were negatively stained with 2% phosphotungstic acid (pH 7.2) on Parlodion-coated grids and examined with a JEOL 100-B transmission electron microscope operated at 60 kV. For each strain tested, at least 100 cells on each of three separate grids were examined for the presence of pili. For observation of capsules, TEM of ultrathin slices stained with ruthenium red was performed as previously described (29, 70).

Cytotoxicity assay. All tissue culture cells were grown in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum and 200 mM L-glutamine at 37°C in 5% CO₂. Cytotoxicity assays were carried out on Chinese hamster ovary cells (CHO) cells, using the procedures outlined by Guerrant et al. (13) that were previously used to measure the cytolysin activity present in supernatants of *V. vulnificus* cultures (65). Briefly, cultures were grown overnight in BHI broth (with chloramphenicol and IPTG where appropriate) at 30°C. Optical densities were measured at 600 nm to verify comparable growth of the cultures. Supernatants were centrifuged twice at 12,000 × g and filtered through 0.2-µm-pore-size cellulose acetate syringe filters to remove bacteria. Serial twofold dilutions were prepared in PBS, and 20 µl of each dilution was added to CHO cell monolayers covered with 200 µl of MEM in 96-well tissue culture plates, each seeded with 4 × 10⁴ CHO cells 24 h prior to the experiment. The cells were then incubated at 37°C in 5% CO₂. Cytotoxicity was determined by changes in CHO cell morphology (cell rounding and lysis; detachment of monolayer from the plastic surface) after 6 h of exposure to the bacterial supernatants. The results are reported as the reciprocal of the lowest dilution causing greater than 75% of the cells to show the altered morphologies.

Tissue culture adherence assay. Quantitative adherence assays were performed on human epidermoid carcinoma cell (HEp-2) monolayers grown in 24-well tissue culture dishes in which each well was seeded with 2 × 10⁵ cells and grown overnight at 37°C in 5% CO₂. The HEp-2 cells were prepared for the assay by removing the medium and washing twice with Hanks' balanced salt solution, followed by the addition of 1 ml of serum-free MEM with Earle's salts. *V. vulnificus* strains were grown overnight in BHI broth (with 5 µg of chloramphenicol per ml and 1 mM IPTG if containing plasmid pMMB67EH.cam or pRPD1) at 30°C with gentle agitation. After measurement of the optical density at 600 nm to estimate bacterial cell concentration from previous growth curves, the cultures were pelleted in a microcentrifuge (6,000 × g), resuspended in PBS to their original concentration, and then diluted in PBS to 4 × 10⁷ CFU ml⁻¹. Cell monolayers were inoculated in triplicate with 50 µl of the diluted bacteria to give a multiplicity of infection (MOI) of ca. 10 and then centrifuged at 670 × g for 10 min at 10°C in an SH3000 rotor. The tissue culture plates were incubated at 37°C in 5% CO₂ for 1 h, and then the monolayers were washed six times with PBS to remove nonadherent bacteria. Following the last wash, the cells were covered with 1 ml PBS and mechanically agitated by vigorous pipetting to suspend epithelial cells and bacteria, followed by serial 10-fold dilutions and quantitation by plating on LB agar. The assay results are presented as the percent cell-associated bacteria, which equals (CFU recovered/CFU inoculated) × 100.

Animal studies. Female, 6- to 8-week old Swiss Webster mice (DNK Universal Inc., Kent, Wash.) were used in all experiments. Intramuscular injections of 5 mg of iron dextran (Sigma, St. Louis, Mo.) in 100 µl of water were administered 2 h prior to challenge as previously described (66). Overnight cultures of bacteria grown in BHI broth with chloramphenicol and IPTG, where appropriate, were pelleted and washed once in PBS prior to resuspension in PBS to appropriate concentrations. The mice (*n* = 5 for each dilution) were inoculated intraperito-

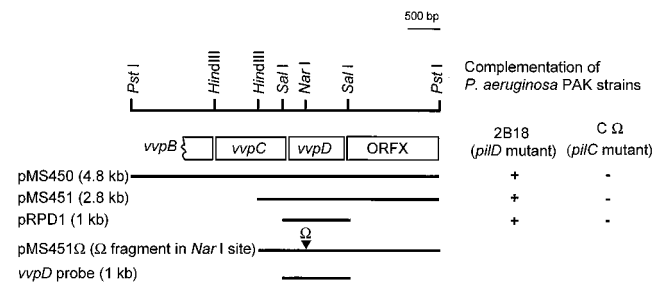


FIG. 1. Genetic organization and partial restriction map of the 4.8-kb *PstI* fragment showing restriction sites used for cloning various constructs. Genes encoded on the fragment are shown as boxes. The ▼ symbol indicates the insertion site for the Ω fragment. Complementation of *P. aeruginosa pilD* and *pilC* mutants, indicated by restoration of PO4 phage sensitivity, is shown on the right. The *vvpD* probe derived from the 1-kb *SalI* fragment used in Southern blots is shown as a bar at the bottom.

neally with 200 µl of the bacteria, and the experiment was terminated after 26 h. The LD₅₀ was calculated by the method of Reed and Muench (46).

Nucleotide sequence accession numbers. The nucleotide sequences of *vvpC* and *vvpD* from both MO6-24 and C7184 have been submitted to the GenBank/EMBL/DBJ data libraries under accession no. U48808 and AF070934, respectively.

RESULTS

Isolation of a *V. vulnificus vvpD* clone that complements a *P. aeruginosa pilD* mutation. A *V. vulnificus* genomic library from strain MO6-24 was conjugated into *P. aeruginosa* PAK 2B18, which contains a Tn5 insertion in the *pilD* gene (36). Clones complementing the *P. aeruginosa pilD* defect were identified by the reappearance of twitching motility (8), which is caused by the polymerization and retraction of type IV pili and is observed in *P. aeruginosa* as characteristic rough and irregular spreading colonies on solid surfaces. Mutants that lack PilD produce small round colonies with smooth edges because the type IV pilin subunits are not processed and assembled into functional pili (36).

Three clones with a twitching motility phenotype, observed as a characteristic rough and irregular spreading morphology, were tested for susceptibility to the *P. aeruginosa* pilus-specific bacteriophage PO4. All PO4-sensitive clones contained similar cosmids, as evidenced by restriction endonuclease digests. One clone, which carried approximately 25 kb of the *V. vulnificus* chromosome, was chosen for further study and designated pJCL12B.

Various restriction fragments of plasmid pJCL12B were subcloned and religated into pLAFR3 in order to delimit the region containing the *V. vulnificus pilD* homolog (Fig. 1). Digestion with *PstI* produced a 4.8-kb fragment that complemented the *P. aeruginosa pilD* mutation (pMS450). The 4.8-kb *PstI* fragment contained two internal *HindIII* sites that permitted its subcloning as three separate fragments in pLAFR3: pMS451 carrying a 2.8-kb *HindIII-PstI* fragment, pMS452 carrying a 1.2-kb *PstI-HindIII* fragment, and pMS453 carrying a 0.8-kb *HindIII* fragment. Only pMS451 complemented the *pilD* mutation in *P. aeruginosa* PAK 2B18, as confirmed by phage PO4 sensitivity.

Sequence analysis of *V. vulnificus vvpC* and *vvpD*. Nucleotide sequencing of the *PstI* fragment from pMS450 (strain MO6-24) revealed the presence of one partial and three complete open reading frames (ORFs) (Fig. 1). Comparison of the ORFs to the GenBank database by using BLAST showed that three of the ORFs encoded proteins homologous to type IV pilus biogenesis proteins previously identified as PilBCD from *P. aeruginosa* (36) and TapBCD from *A. hydrophila* (40); the ORF

Vv VvpC (C7184)	MLKLT	HSQIKNYQWK	GINGAGKKVS	GNTLALTELE	VRDLKMQHI
Vv VvpC (MO6-24)	MLKLT	HSQIKNYQWK	GINGAGKKVS	GNTLALTELE	VRDLKMQHI
Pa PilC	MADK	ALRTSVFTWE	GTDKGAKRVS	GELTQGMFL	VKALHRKQGI
Ah TapC	MATLTQKQNA	PKKVFAFRWS	GVNRGQKVS	GELQADSIMT	VKAEHLRQGV
50					
Vv VvpC (C7184)	QIKKIKKSSV	SVFTRLSHKV	KGKDTLLTR	QLATMLPTGV	PILQSLKVA
Vv VvpC (MO6-24)	QIKKIKKSSV	SVFTRLSHKV	KGKDTLLTR	QLATMLPTGV	PILQSLKVA
Pa PilC	NFLKVKKGI	SLIGL	AGKVK	KPMDIALFTR	QMATMAGAGV
Ah TapC	NVTKVSQK	GLRFSKGAKI	KPMDIAIVSR	QITTMLSAGV	PLVQSLQIIA
100					
Vv VvpC (C7184)	DNHRKAEMKS	ILANVSKAVE	AGTPLSKALR	SASPOFDGIY	VDLVATGQES
Vv VvpC (MO6-24)	DNHRKAEMKS	ILANVSKAVE	AGTLLSKALR	SASPOFDGIY	VDLVATGQES
Pa PilC	EGFDNPNMRK	LVDEIKQEV	SGNSLANSR	KPKQYDFDELY	CNLVDAGEQS
Ah TapC	RSHEKASMRE	LMQGLAADVE	TGPFMSALR	RHRPHFDALY	CDLVEAGEQS
150					
Vv VvpC (C7184)	GNLDQVFERL	AFYREKNEQL	RAKVIKALIY	PCMVVLVALG	VSYLMLTQCY
Vv VvpC (MO6-24)	GNLDQVFERL	AFYREKNEQL	RAKVIKALIY	PCMVVLVALG	VSYLMLTQCY
Pa PilC	GALENLDRV	ATYKERTESL	KAKIKKAMTY	PIAVLIVALI	VSAILLIK.V
Ah TapC	GALETTYDRI	ATYREKSEAL	KSKIKKAMFY	PAMVILVAIV	VTSILLFL.V
200					
Vv VvpC (C7184)	IPNFARCSPR	FGAELPWFTQ	KVLDSLDFVQ	AYSSWFFITL	FSAIYLLRGL
Vv VvpC (MO6-24)	IPNFARCSPR	FGAELPWFTQ	KVLDSLDFVQ	AYSSWFFITL	FSAIYLLRGL
Pa PilC	VFQFQSVFEG	FGAELPAFTQ	MIVNLSDFVQ	EWFFILLAI	AIFGFAPREL
Ah TapC	IFQFEDIFKS	FGAELPIFTQ	FVIGISRFVQ	HWVYVIFGTT	AFAPILYVRA
250					
Vv VvpC (C7184)	SRKSPAFRLK	LSRLSLKIPV	IGGVLSKAAI	AKFSRTLATS	FSAGIPILTS
Vv VvpC (MO6-24)	SRKSPAFRLK	LSRLSLKIPV	IGGVLSKAAI	AKFSRTLATS	FSAGIPILTS
Pa PilC	HKRSQKFRDT	LDRTILAKLP	FGGIVYKSAV	ARYARTLSTT	FAAGPLVDA
Ah TapC	WRASQKVDN	TDFKLTITPV	VGMIHLKAA	ARFARTLSTT	FAAGPLVDA
300					
Vv VvpC (C7184)	LKTTSTKTAGN	LHYQYAIEDV	YRDTAAGMPM	YIAMRNSLAF	PELVQMVM
Vv VvpC (MO6-24)	LKTTSTKTAGN	LHYQYAIEDV	YRDTAAGMPM	YIAMRNSLAF	PELVQMVM
Pa PilC	LDSVSGATGN	LVFKNAVSKI	KQDVSTGMQL	NFSMRITTSV	PNMAIQMTAI
Ah TapC	LISAAGASGN	VYRYTAVMAI	RNEVVGAMQI	NVAMRTVDLF	PDVVIQVM
350					
Vv VvpC (C7184)	GEESGQMDM	LNKIATIYEF	EVDTVDNLG	KILEPLIIVF	LGVVGGVLT
Vv VvpC (MO6-24)	GEESGQMDM	LNKIATIYEF	EVDTVDNLG	KILEPLIIVF	LGVVGGVLT
Pa PilC	GEESGSLDEM	LSKVASIYEE	EVDNAVDNLT	TLMEPFIMAV	LGVVGGVLT
Ah TapC	GEESGALDDM	LSKVIATIFEQ	EVDDLVDGLT	SILLEPLIMV	LGVVGGVLT
400					
Vv VvpC (C7184)	AMYLPIFNLM	SVLG			
Vv VvpC (MO6-24)	AMYLPIFNLM	SVLG			
Pa PilC	AMYLPIFQLG	HWVG			
Ah TapC	AMYLPIFKLG	DLVG			
414					

FIG. 2. Comparison of amino acid sequences of *V. vulnificus* VvpC (Vv VvpC) with homologs from *P. aeruginosa* (Pa PilC) and *A. hydrophila* (Ah TapC). Differences in the C7184 and MO6-24 VvpC sequences are shaded. The alignment was generated by using the Pileup program of the Genetics Computer Group (Madison, Wis.).

products and their corresponding genes were designated VvpBCD and *vvpBCD* (*V. vulnificus* pili). The first partial ORF product had strong homology to the ABC transporter-like PilB/TapB. A second clone has been isolated since the initial isolation of pJCL12B that contains a complete *vvpB* gene; its characterization will be reported elsewhere. There was a complete ORF immediately downstream of the partial *vvpB* gene, designated *vvpC*, encoding a protein of 409 amino acids with strong homology to PilC/TapC, a protein of unknown function but absolutely required for pilus assembly (36). VvpC shows 55% similarity and 41% identity to PilC and 58% similarity and 45% identity to TapC (Fig. 2).

Downstream and adjacent to *vvpC* is *vvpD*, which encodes a 289-amino-acid protein that shows 59% similarity and 52% identity to PilD and 62% similarity and 55% identity to TapD (Fig. 3). The sequence also reveals the presence of two pairs of cysteine residues, at positions 70 and 73 and positions 95 and 98, and an invariant glycine at position 92, which in *P. aeruginosa* (PilD) have been shown to be important in leader peptide cleavage and methyltransferase activities of the enzyme (41, 55). Immediately downstream of the *vvpD* gene is a third ORF encoding a 22.5-kDa protein highly homologous to products of several similarly sized ORFs of unknown function, located downstream of *pilD* homologs in *A. hydrophila*, *Pseudomonas putida*, and *Xanthomonas campestris* (40).

During this work we found that allelic exchange was more efficient in *V. vulnificus* C7184 than in MO6-24. Therefore, a DNA fragment containing the genes encoding *vvpC* and *vvpD* from strain C7184 was generated by PCR using primers specific for the flanking regions of MO6-24 *vvpC* and *vvpD*, as described in Materials and Methods. Sequencing of the PCR-generated C7184 *vvpD* showed it to be identical to *vvpD* from MO6-24. There were some differences in the nucleotide sequences of *vvpC* from MO6-24 and C7184 that result in a Leu-

Vv VvpD	MDALQY.	YFWLYIGLAS	LFGLLVGSFL	NVVYIRLFI	MELEWRQCEA
Ah TapD	MALLELEAHG	LFWLYFSLVF	LFSLMIGSFL	NVVYIRLFI	LEREWQAEYR
Pa PilD	MFLLDYLAS.	HPLAFVLCAT	LLGLLVGSFL	NVVYIRLFI	MERNWKAER
50					
Vv VvpD	ESFPEYNTPT	PTETLTSTP	RSSCPSCHTP	TRVRDNIPVF	SWLALRQKCH
Ah TapD	SYFNPDDRGV	DEPPYINLMP	RSCCPCHNHP	ITALENIPLL	SWLWLRQRCR
Pa PilD	EALGLEPE.P	KQATYNLVL	NSACPCRQHE	IRPWENIPLV	SYLALGGKCS
100					
Vv VvpD	HCQTKISARY	PFVEALSAPL	CGLVAMKFGY	APITVALIGF	TLVLIATFTI
Ah TapD	GCQAPISARY	PIVELLTALL	SVAVANTLAP	GWGTLAALLL	TWLVVALFTI
Pa PilD	SCKAATGKRY	PIVELATALL	SGVAVMHPGF	TWQAGAMLLL	TWGLLAMSLL
150					
Vv VvpD	DLDTMLLPDQ	LTLPLTWGTI	ALALLEISPV	SLQDSVFAGM	AGYLCVLSVY
Ah TapD	DLDKMLLPDQ	LTLPLTWGGL	LFNLLGGF.V	SLGDVIGAM	AGYLVLSVLY
Pa PilD	DADHQLLPDV	LVLPLLWGL	TANHFGLF.A	SLDDALFGAV	FGYLSLWSVF
200					
Vv VvpD	HLFRLLTGKE	GMGYGDFKLL	AALGAWLQWQ	YLFMIILLSS	VVGLIFGLIQ
Ah TapD	WAFKLLTGKE	GMGYGDFKLL	AALGAWLQWQ	ALPIVLLSS	LVGAFMGLQ
Pa PilD	WLFKLVGTKE	GMGYGDFKLL	AMGAWGQWQ	ILPITLILLSS	LVGAILGVIM
250					
Vv VvpD	LRLQKQGIEM	APFPGLYLA	AGWVALMWDG	SLMSWYMLYL	IGA
Ah TapD	ILLRNHHQSK	PIPPGLYLA	AGWIALMWDG	SITRWYLTNF	L--
Pa PilD	LRLRNAESGT	PIPPGLYLA	AGWIALMWDG	QITRTYLQFA	GFK
293					

FIG. 3. Comparison of amino acid sequences of *V. vulnificus* VvpD (Vv VvpD) with homologs from *P. aeruginosa* (Pa PilD) and *A. hydrophila* (Ah TapD). The conserved cysteine and glycine residues shown to be involved in leader peptidase and *N*-methyltransferase activities are shaded. The alignment was generated by using the Pileup program of the Genetics Computer Group.

to-Pro substitution at amino acid position 119, and a Ser to Pro substitution at position 176, in the deduced amino acid sequences (Fig. 2).

vvpD is conserved in *V. vulnificus* strains. An environmental isolate, PAC1, was compared with the two clinical isolates of *V. vulnificus*, MO6-24 and C7184, for the presence of the type IV pilus biogenesis gene cluster. Chromosomal DNA from all of three strains was digested with the restriction enzymes *Pst*I, *Pst*I-*Hind*III, and *Sal*I and probed under high stringency with the *vvpD* gene (a 1-kb *Sal*I fragment). The *vvpD* sequence was present on a 1-kb *Sal*I fragment as well as an 11-kb *Pst*I fragment in all three strains (Fig. 4). Probing a *Pst*I-*Hind*III double digest confirmed the presence of *vvpD* on a 2.8-kb fragment in strains MO6-24 and C7184. However, probing PAC1 DNA digested with the same enzymes showed hybridization to a 3.6-kb fragment demonstrating a minor restriction site polymorphism and a missing downstream *Hind*III site (Fig. 1).

V. vulnificus VvpC does not complement *P. aeruginosa* PilC. Both TapC and TapD of *A. hydrophila* can complement their homologous counterparts in *P. aeruginosa* (40), and as shown here, VvpD can functionally replace PilD. To determine whether *V. vulnificus* VvpC can complement PilC, plasmids carrying

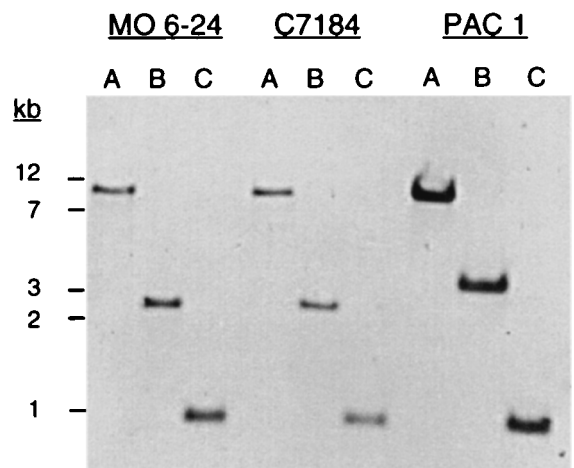


FIG. 4. Southern blot comparison of various restriction digests of *V. vulnificus* chromosomal DNA, using *vvpD* as a probe. Lanes: A, *Pst*I; B, *Pst*I-*Hind*III; C, *Sal*I. Positions of molecular weight markers are indicated on the left.

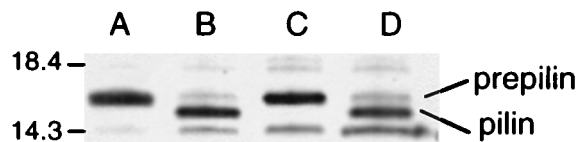


FIG. 5. Sodium dodecyl sulfate-Tricine-15% polyacrylamide gel showing *in vitro* activity of VvpD on *P. aeruginosa* prepilin substrate. Membranes were prepared from *V. vulnificus* strains carrying the plasmids indicated. Lanes: A, prepilin substrate only; B, C7184(pMMB67HE.cam); C, C7184D₁₂Ω(pMMB67HE.cam); D, C7184D₁₂Ω(pRPD1). Positions of molecular weight markers are shown on the left in kilodaltons.

vvpC and *vvpD* from strain MO6-24(pMS450), *vvpD* from strain C7184(pRPD1), and the vector alone (pMMB67HE.cam) were conjugated into *P. aeruginosa* PAK 2B18 and *P. aeruginosa* PAK-CΩ, a *pilC* null mutant (23). Testing for restoration of pilus expression by sensitivity to phage PO4 showed that pMS450 and pRPD1 complemented PAK 2B18 but not PAK-CΩ (Fig. 1). Therefore, while VvpD is expressed and complements its homolog in *P. aeruginosa*, we were unable to demonstrate complementation by VvpC. However, it is unknown whether this is due to any differences in specificity of the proteins or due to lack of expression or incorrect cytoplasmic membrane localization of VvpC in *P. aeruginosa*.

Construction of a *V. vulnificus vvpD* mutant. To construct a *V. vulnificus vvpD* mutant, the 2.0-kb Ω interposon encoding spectinomycin and streptomycin resistance (42) was cloned into a unique *NarI* site within *vvpD* (137 bp downstream of the *vvpD* ATG start codon) in plasmid pMS4514, creating pMS451Ω (Fig. 1). The 4.8-kb DNA fragment containing *vvpD*::Ω was then subcloned into the suicide vector pEP185.2 (20) as a *SmaI*-*KpnI* fragment and maintained in *E. coli* S17-1λpir (32, 50). This construct (pMS451Ω-2) was subsequently transferred into *V. vulnificus* C7184 by conjugation. Double recombinants were selected for spectinomycin resistance and screened for chloramphenicol sensitivity, and then the presence of only the insertionally inactivated copy of *vvpD* was confirmed by Southern blot analysis (data not shown). One strain, C7184D₁₂Ω was selected for further study.

VvpD is a type IV leader peptidase. Complementation of the *P. aeruginosa pilD* mutation by *vvpD* demonstrates that VvpD can replace the type IV peptidase activity of PilD. To confirm that VvpD acts to cleave the leader sequence from a type IV prepilin, an *in vitro* assay was performed. Plasmids pRPD1 and pMMB67HE.cam were conjugated into C7184 and C7184D₁₂Ω. Membrane fractions prepared from these strains were incubated with *P. aeruginosa* purified prepilin as the substrate (59). The prepilin incubated with membranes from C7184(pMMB67HE.cam) and C7184D₁₂Ω(pRPD1) show an increased mobility, suggesting cleavage of the leader peptide from the prepilin. There was no shift in the mobility of the prepilin band treated with membranes derived from C7184D₁₂Ω(pMMB67HE.cam) compared to prepilin alone, indicating the absence of a functional type IV peptidase (Fig. 5). These mobility shifts were identical to those seen with PilD-containing membranes as a source of enzyme activity (data not shown). Although this experiment does not unequivocally show that VvpD cleaves the leader peptide precisely at the site cleaved by PilD (MKAQ KG[▼]FTLIE, where [▼] is the cleavage site), sufficient evidence has accumulated through N-terminal amino acid sequencing that expression of pili *in vivo* requires that the leader sequence of the *P. aeruginosa* prepilin (and pilin-like proteins of the type II secretion system) be cleaved between these glycine and phenylalanine residues before polymerization of the pilin monomers into pili can proceed (37, 60).

The *vvpD* mutation defines a type II secretion system in *V. vulnificus*. In addition to pilus biogenesis, *P. aeruginosa* PilD has been shown to be required for extracellular protein secretion via the GSP or type II pathway (43, 58). To determine the role of VvpD in protein secretion, we compared the activities and localization of three enzymes, the cytolysin/hemolysin, protease, and chitinase. Cells from C7184(pMMB67HE.cam), C7184D₁₂Ω(pMMB67HE.cam), and C7184D₁₂Ω(pRPD1) were fractionated to compare the activities of the cytolysin in the cytoplasm, periplasmic space, and extracellular milieu. Activity of the cytolysin was measured by its hemolytic titer in culture filtrates of cell fractions. In C7184(pMMB67HE.cam), all activity was in the supernatant fraction; none was detected in either the periplasmic or cytoplasmic fraction (Table 2). The majority of the hemolytic activity was concentrated in the periplasmic fraction in C7184D₁₂Ω(pMMB67HE.cam), with some residual activity in both the supernatant and cytoplasmic fractions, indicating a defect in secretion of the cytolysin from the periplasm across the outer membrane. The defect in secretion was restored in the complemented strain, C7184D₁₂Ω(pRPD1), where the majority of the cytolysin was detected in the supernatant fraction. Cytolysin was not detected in any of the cell fractions of a cytolysin (*vvhA*) mutant, C7184-402K, which was included as a control.

The same strains were compared for extracellular secretion of protease and chitinase. C7184(pMMB67HE.cam) and C7184D₁₂Ω(pRPD1) secreted protease extracellularly, but secretion of protease was not observed in the mutant strain, C7184D₁₂Ω(pMMB67HE.cam) (Table 2). Extracellular secretion of chitinase by C7184(pMMB67HE.cam) and C7184D₁₂Ω(pRPD1) was confirmed by a zone of clearing around the area of inoculation on plates containing chitin. The mutant strain C7184D₁₂Ω(pMMB67HE.cam) showed no secretion of chitinase (Table 2).

Inactivation of VvpD results in loss of surface pili. Transmission electron micrographs of wild-type C7184 show the presence of fine hairlike structures of typical size and length for type IV pili (57) extending from the surface of the cell (Fig. 6A). Pili were not detected on the surface of C7184D₁₂Ω (Fig. 6B), indicating that a mutation in *vvpD* results in loss of expression of pili on the surface of the cells. When VvpD function was restored in the complemented strain C7184D₁₂Ω(pRPD1), pili were again observed on the surface of the cells (Fig. 6C). Greater than 50% of the wild-type and complemented mutant cells were piliated as visualized by TEM, while no surface or background pili were observed on the mutant alone.

Loss of VvpD does not affect serum resistance and encapsulation of *V. vulnificus*. The specific virulence functions attributed to the *V. vulnificus* capsule include resistance to the bactericidal effects of serum as well as inhibition of phagocytosis

TABLE 2. Secretion of cytolysin, protease, and chitinase in *V. vulnificus*

Strain	Hemolytic index ^a			Extracellular secretion ^b	
	Super-natant	Peri-plasm	Cyto-plasm	Pro-tease	Chiti-nase
C7184(pMMB67HE.cam)	2,048	0	0	+	+
C7184D ₁₂ Ω(pMMB67HE.cam)	8	272	4	-	-
C7184D ₁₂ Ω(pRPD1)	2,048	16	4	+	+
C7184-402K	0	0	0	ND	ND

^a Reciprocal of the highest dilution of culture supernatant or cell fraction at which hemolysis was observed (average of two assays).

^b Detected by a zone of clearing around the bacterial colonies (see Materials and Methods). ND, not done.

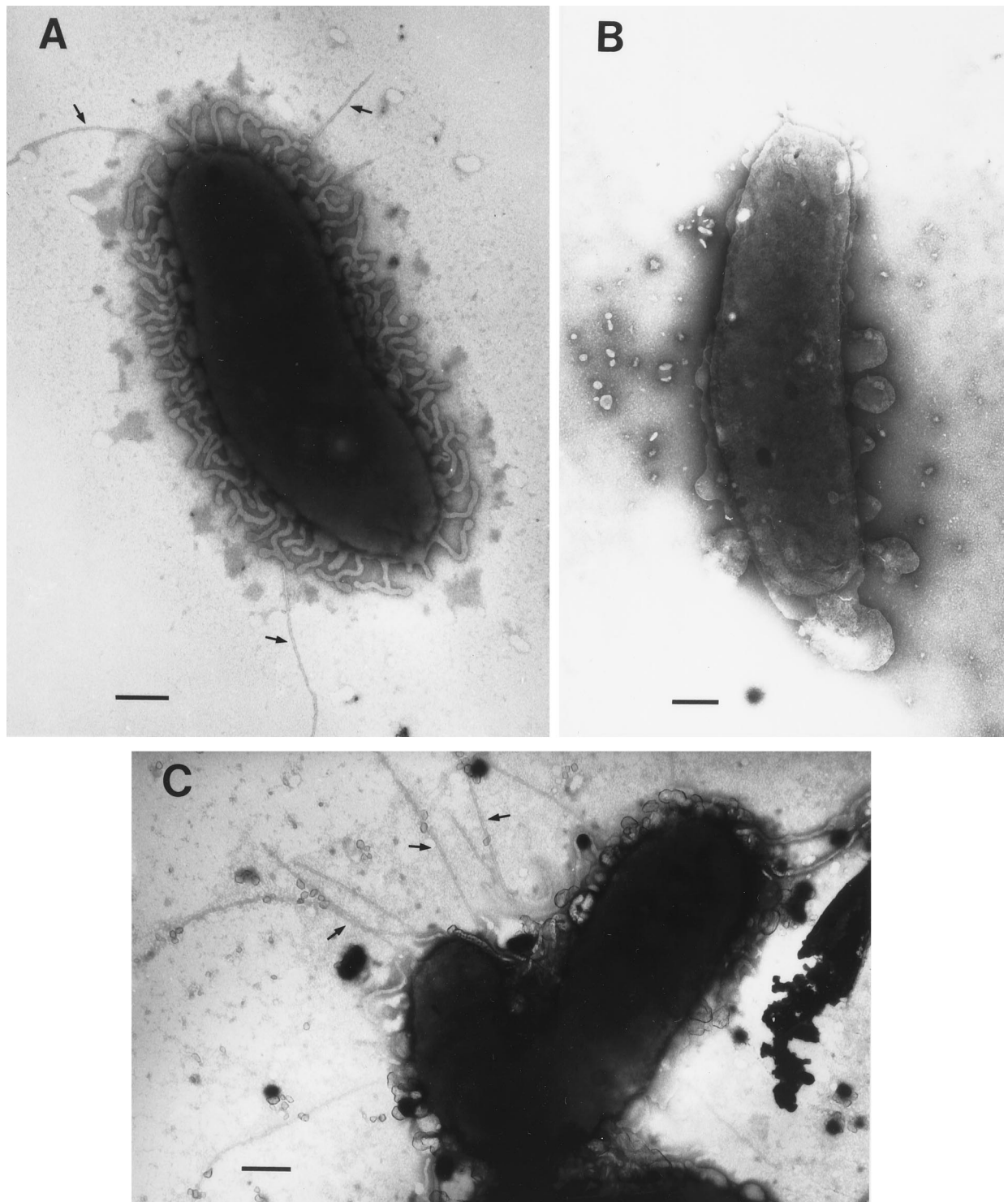


FIG. 6. Electron micrographs of *V. vulnificus* C7184 strains showing surface pili (indicated by arrows). (A) C7184; (B) C7184D₁₂Ω; (C) C7184D₁₂Ω(pRPD1). Bar = 200 nm.

by macrophages. To determine whether *V. vulnificus* C7184D₁₂Ω is equally or less serum resistant than wild-type C7184, a serum sensitivity assay was performed. As shown in Table 3, the wild-type and *vvpD* mutant strains of C7184 were equally resistant to the bactericidal effect of human serum at levels significantly higher than the serum-sensitive *E. coli* S17-1λpir.

The presence of an intact polysaccharide capsule was then confirmed. Whereas unencapsulated variants of *V. vulnificus* are translucent in visible light (67), colonies of *V. vulnificus* C7184D₁₂Ω are opaque and indistinguishable from wild-type colonies, suggesting no alteration of capsule expression. This was experimentally confirmed by demonstrating there was no

TABLE 3. Serum resistance

Strain	Serum resistance ^a	
	Normal	Heat inactivated
<i>V. vulnificus</i>		
C7184	0.46	0.44
C7184-D ₁₂ Ω	0.93	0.65
<i>E. coli</i> S17-1λpir	>6.4	0.71

^a Log₁₀ reduction of bacterial numbers before and after incubation for 1 h at 37°C in normal or heat-inactivated serum.

difference in hydrophobicity in ammonium sulfate (67) between C7184 and C7184D₁₂Ω (data not shown). Finally, electron micrographs (Fig. 7) of ruthenium red-stained ultra thin sections show that both the wild-type and *vvpD* mutant strains have an electron-dense layer outside their outer membranes typical of encapsulated *V. vulnificus* (67).

VvpD is required for culture supernatant cytotoxicity on CHO cells. Culture supernatants from *V. vulnificus* are cytotoxic to cultured epithelial cells, an activity attributed to the cytotoxin, a 51-kDa protein that is hemolytic to mammalian erythrocytes. Purification and subsequent cloning and sequencing indicated that this cytotoxic and hemolytic activity was caused by the protein, designated VvhA or cytotoxin, which has some homology to the *V. cholerae* El Tor hemolysin (12, 65, 68). To determine the effect of the *vvpD* mutation on cytotoxicity, serial two-fold dilutions of filtered supernatants from overnight cultures were added to monolayers of CHO cells, incubated for 6 h at 37°C, and monitored for cell rounding and destruction of the monolayer (65). As expected from the results obtained in the cytotoxin localization assays, the supernatant from C7184D₁₂Ω was able to cause a cytopathic effect only at the highest concentration, while the supernatant from wild-type C7184 had to be diluted more than 64-fold before no effect on the CHO cells was observed (Table 4). Complete cytotoxicity of C7184D₁₂Ω was restored by complementation with pRPD1. Surprisingly, the supernatant from strain C7184-402K, which contains a kanamycin resistance cassette in *vvhA* and does not express the cytotoxin, is equally cytotoxic to CHO cells (Table 4), contrary to results reported previously (64). This suggests that cytotoxicity of culture supernatants from *V. vulnificus* is due to a combination of several exported degradative and cytotoxic en-

zymes, perhaps acting in concert. However, the role of VvhA is unclear.

VvpD is required for HEp-2 cell adherence. To determine if pili or other type II secretion products play a role in virulence of *V. vulnificus* by aiding adherence to epithelial cells, we developed an adherence assay that is more quantitative and reproducible than the method previously used (11). The assay involves adding washed *V. vulnificus* cells to monolayers of HEp-2 cells at numbers no greater than 10 CFU per epithelial cell. The low MOI prevents the rapid cytotoxic effect that destroys the integrity of the tissue culture monolayer when higher MOIs are used. Adherent bacteria are resuspended by mechanical agitation instead of lysing the epithelial cells with detergents such as Triton X-100 because *V. vulnificus* is extremely sensitive to rapid lysis by these agents as well. Following the procedure outlined in the Materials and Methods, ca. 9% of wild-type strain C7184(pMMB67HE.cam) input bacteria remained adherent to the HEp-2 cell monolayer after extensive washing (Table 4). C7184D₁₂Ω(pMMB67HE.cam) was consistently and significantly less adherent than the wild-type parent strain, with less than 0.5% of input bacteria remaining associated with the monolayers. Complementation of C7184D₁₂Ω with pRPD1 significantly increased adherence to a value about half of that for the wild-type strain, suggesting that adherence of *V. vulnificus* to HEp-2 cells requires pili, a type II secretion pathway product (or products), or both. Adherence was not dependent on the presence of VvhA, as strain C7184-402K was as adherent to HEp-2 cells as the wild-type C7184 strain.

Virulence is dependent on VvpD. The LD₅₀s in iron-overloaded mice after intraperitoneal injections of *V. vulnificus* strains are shown in Table 4. Strain C7184D₁₂Ω showed more than a 2-log increase in LD₅₀ over the wild-type strain. This difference is not attributed to lack of or slower growth of the mutant strain *in vivo*. In limited analyses, 15- to 30-fold more CFU than initial challenge inocula were recovered 24 h postinfection from homogenized spleens of mice whether they were infected with the wild-type, the mutant, or the complemented mutant strain. Complementation of C7184D₁₂Ω with *vvpD* *in trans* on plasmid pRPD1 partially restored virulence to a value 1 log less than that for the uncomplemented mutant. Also, at inoculum levels that eventually resulted in the death (within 26 h) of all five mice injected with C7184D₁₂Ω(pMMB67HE.cam) or C7184D₁₂Ω(pRPD1), the time to death for the mice infected with the latter was consistently 2 to 4 h less than for

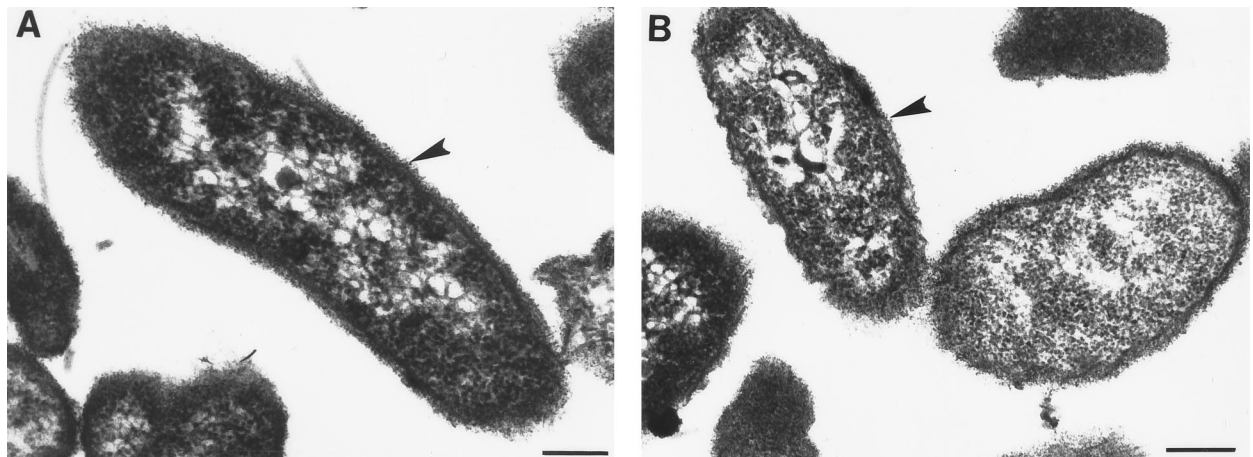


FIG. 7. Electron micrographs of *V. vulnificus* C7184 (A) and C7184D₁₂Ω (B) stained with ruthenium red. Bar = 200 nm.

TABLE 4. Cytotoxicity, adherence, and virulence

<i>V. vulnificus</i> strain	CHO cell cytotoxicity ^a	HEp-2 cell adherence (%) ^b	Mouse LD ₅₀
C7184(pMMB67HE.cam)	64	9.2 ± 2.1	9.4 × 10 ³
C7184-D ₁₂ Ω(pMMB67EH.cam)	<2	0.3 ± 0.1 ^c	1.1 × 10 ⁶
C7184-D ₁₂ Ω(pRPD1)	64	4.5 ± 2.0 ^c	2.1 × 10 ⁵
C7184-402K	64	9.0 ± 3.6	ND ^d

^a Reciprocal of the highest dilution of filtered bacterial supernatants to cause typical CHO cell cytotoxicity (cell lysis, rounding, and detachment of monolayer) after 6 h.

^b Presented as (number of CFU recovered/number of input CFU) × 100 ± standard deviation.

^c Significantly different when compared by Tukey-Kramer analysis at $P \leq 0.5$.

^d ND, not done.

the uncomplemented mutant (data not shown). Again, equal numbers of bacteria were recovered from spleens of infected animals regardless of the strain or plasmid construct it carried. However, it was found during these analyses that the plasmid containing *vvpD* was not maintained in these strains in the absence of antibiotic selection and was rapidly lost within 24 h postinfection by approximately 99% of the bacteria during *in vivo* cell division. This may account for the partial complementation in C7184D₁₂Ω(pRPD1) to an LD₅₀ lower than that of the wild type.

DISCUSSION

With the exception of the polysaccharide capsule, it has been difficult to identify specific factors that contribute to the pathogenicity of *V. vulnificus*. The data presented in this report show that a mutation in a single gene encoding a type IV peptidase/*N*-methyltransferase in *V. vulnificus* causes pleiotropic defects in expression of pili and in the type II extracellular protein secretion pathway. These defects significantly decrease tissue culture adherence and cytotoxic activity, as well as virulence in the iron-overloaded mouse model. The *in vitro* results obtained with tissue culture experiments suggest that specific colonization factors such as type IV pili are required for adherence of *V. vulnificus* to epithelial cells. In addition, this mutant is significantly less cytotoxic to cultured epithelial cells than the wild-type or the cytolysin mutant, suggesting that multiple secreted products and possibly close contact mediated by the pili are necessary for cytotoxicity. Since the *vvpD* mutation has no measurable effect on capsule expression, these results indicate that a combination of pili and/or secreted protein(s) may play a significant role in *V. vulnificus* pathogenesis, as indicated by the reduced virulence in mice. Clarification of these possibilities will be aided by the construction of a mutant unable to express the type IV pilin subunit, the gene for which has recently been localized upstream of *vvpC* and *vvpD* (unpublished data).

V. vulnificus produces a number of degradative and cytotoxic enzymes that are important for survival in its normal environmental niche, namely, estuarine waters and molluscan shellfish. A number of these, including the cytolysin, an elastolytic protease (24), and an enterotoxin (54), have been suggested to be important for the rapid invasiveness, dissemination, and tissue damage caused by the organism after ingestion of contaminated shellfish or infections through wounds. Adherence to cells mediated by pili has also been suggested to be an important virulence determinant (11).

The purified cytolysin protein is cytotoxic to CHO cells, is hemolytic for mammalian erythrocytes, causes vascular permeability in guinea pig skin, and is lethal for mice (12, 25). De-

tection of specific anticytolysin antibody in convalescent sera suggests that it is expressed during clinical infection. However, its contribution to virulence is suspect in that a cytolysin mutant strain of *V. vulnificus* is just as virulent in the mouse model as isogenic wild-type strains (64). It is unclear why the mutant strain that we constructed by allelic exchange of wild-type *vvhA* with a copy interrupted with a kanamycin resistance cassette is still cytotoxic to CHO cells, even though the cytolysin (hemolysin) assays clearly show that VvhA is not expressed (Table 2). The *vvhA* mutant strain previously reported to lack CHO cell cytotoxicity (64) contains a transposon Tn10 insertion in *vvhA*. It is possible the Tn10 insertion had a polar effect on the expression of an adjacent gene(s) required for tissue culture cytotoxicity in addition to cytolysin. Interestingly, cytotoxicity of a nonhemolytic *vvhA* mutant (constructed by allelic exchange of *vvhA* with a copy insertionally inactivated by a non-transposon antibiotic resistance cassette) similar to our C7184-402K was not reported in this same study (64).

To our knowledge, there has been only one other report describing adherence of *V. vulnificus* to cultured epithelial cells (11). In that study, Gander and LaRocco described the presence of surface pili on a variety of *V. vulnificus* strains and reported that clinical isolates from blood or wounds of infected individuals averaged higher numbers of individual pili per cell than environmental isolates. In assays where they directly enumerated adherent bacteria on monolayers of HEp-2 cells, they correlated adherence with this increased piliation. Environmental isolates, on average, showed fewer bacteria adhering to individual epithelial cells. We are interested in determining whether the quantitative assay that we developed to measure bacterial adherence will show similar differences between environmental (i.e., from oysters) and clinical (blood or wound) isolates.

In addition to the role of pili, other virulence factors have been examined in an attempt to determine why various *V. vulnificus* strains differ in pathogenicity. Oysters are colonized with up to hundreds of individual *V. vulnificus* strains when differentiated by clamped field electrophoresis, pulsed-field gel electrophoresis, or ribotyping (9, 61), and mouse bioassays demonstrate that there are virulent and avirulent strains (53, 62). However, evidence has accumulated to suggest that human infections are caused by only a few of the strains present in the heterogeneous populations found in shellfish (16). To account for these differences in virulence, studies have focused on the cytolysin (18, 62), serum resistance (19, 53), and agglutinating property of the capsule (14). To date a definitive phenotypic difference has not been demonstrated between virulent and less virulent strains, with the exception that loss of capsule correlates with loss of virulence (49, 67, 70).

We have demonstrated the role of the type IV peptidase/*N*-methyltransferase VvpD from *V. vulnificus* in the expression of pili and extracellular secretion by the GSP or type II pathway. Pili and/or one or more of the secreted factors appear to be required for tissue culture cytotoxicity, cell adherence, and virulence. These results indicate that in addition to the capsule, *V. vulnificus* expresses additional virulence determinants that possibly could be targeted for therapeutic intervention.

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