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# A *Vibrio vulnificus* Type IV Pilin Contributes to Biofilm Formation, Adherence to Epithelial Cells, and Virulence

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***Vibrio vulnificus*** **expresses a multitude of cell-associated and secreted factors that potentially contribute to pathogenicity, although the specific roles of most of these factors have been difficult to define. Previously we have shown that a mutation in *pilD* (originally designated *vvpD*), which encodes a type IV prepilin peptidase/*N*-methyltransferase, abolishes expression of surface pili, suggesting that they belong to the type IV class. In addition, a *pilD* mutant exhibits reduced adherence to HEp-2 cells, a block in secretion of several exoenzymes that follow the type II secretion pathway, and decreased virulence. In this study, we have cloned and characterized a *V. vulnificus* type IV pilin (PilA) that shares extensive homology to group A type IV pilins expressed by many pathogens, including *Vibrio cholerae* (PilA), *Pseudomonas aeruginosa* (PilA), and *Aeromonas hydrophila* (TapA). The *V. vulnificus pilA* gene is part of an operon and is clustered with three other pilus biogenesis genes, *pilBCD*. Inactivation of *pilA* reduces the ability of *V. vulnificus* to form biofilms and significantly decreases adherence to HEp-2 cells and virulence in iron dextran-treated mice. Southern blot analysis demonstrates the widespread presence of both *pilA* and *pilD* in clinical as well as environmental strains of *V. vulnificus*.**

*Vibrio vulnificus*, a halophilic, estuarine bacterium, is an opportunistic human pathogen capable of causing fatal primary septicemias or necrotizing wound infections (60). Primary septicemia usually occurs in susceptible hosts following ingestion of raw seafood, mainly shellfish, which are often naturally colonized by the organism. Wound infections are usually acquired through introduction of the organism into preexisting or new wounds, often during handling of shellfish harvested from waters where the organism is present. Host risk factors that contribute to susceptibility include liver disorders such as cirrhosis and alcoholic liver disease, hematological conditions such as hemochromatosis, and compromised immune status (60).

A number of individual bacterial surface factors and extracellular proteins have been implicated in the virulence and pathogenesis of *V. vulnificus*, including an exopolysaccharide capsule, lipopolysaccharide, metalloprotease, cytolysin, phospholipase, and siderophores (reviewed in reference 60). Construction of specific mutations in genes controlling expression of these cell-associated and extracellular factors and analysis of virulence in animal models have shown that only the exopolysaccharide capsule, the presence of a functional flagellar biogenesis system, and the siderophore to acquire iron from transferrin are essential virulence determinants (28, 74, 77). A determination of the in vivo role of other factors, which in general have potent in vitro activities, has remained elusive (16, 49, 72). Increasingly, experimental evidence suggests that virulence of *V. vulnificus* is multifactorial (60) and that the coordinate regulation and expression of several virulence factors are likely controlled by global regulatory systems such as the LuxS quorum-sensing system, the ToxR regulatory system, and the general stress regulator, *rpoS*-encoded sigma factor (15, 20, 27, 31).

In a previous study, we described the isolation of genes encoding factors involved in biogenesis of type IV pili and type II secretion of extracellular proteins (40). A mutation in the gene that encodes a type IV prepilin peptidase/*N*-methyltransferase results in loss of surface pili and extracellular secretion of several enzymes, including cytolysin, metalloprotease, and chitinase, as well as a significant reduction in virulence. This gene—which we initially designated *vvpD* but will now call *pilD* to follow a more standardized nomenclature (10, 36, 67) and to avoid confusion with the subsequent designation of the *V. vulnificus* metalloprotease as VVP (16, 27)—was shown to be located immediately downstream of two other genes encoding proteins homologous to type IV pilus biogenesis factors, *pilB* and *pilC*. In this study, we report the completion of sequencing and characterization of a *pilABCD* gene cluster similar to those found in other gram-negative bacteria such as *Pseudomonas aeruginosa* (36), *Aeromonas hydrophila* (41), and *Vibrio cholerae* (10, 67). The role of PilA in expression of pili, adherence to epithelial cells, biofilm formation, and virulence in *V. vulnificus* was also assessed.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** The bacterial strains and plasmids used in this study are listed in Table 1. *V. vulnificus* strains were grown at 30°C in Luria-Bertani (LB) broth. In addition, brain heart infusion broth (BHI), Trypticase soy broth (TSB), Marine broth, and TCG broth (1% Bacto Tryptone, 0.3% yeast extract, 0.5% NaCl, 0.3% $NaHCO_3$, 0.02% thioproline, 0.1% L-glutamic acid monosodium salt, 1 mM EGTA) (6) were used as noted. When modified TCG broth was used, EGTA was omitted from the medium. *Escherichia coli* strains were grown at 37°C in LB broth. Antibiotics were used at the following concentrations: chloramphenicol, 30 μg/ml; streptomycin, 25 μg/ml; spectinomycin, 25 μg/ml; and ampicillin, 50 μg/ml. Polymyxin B was used at 50 U/ml. Isopropyl -β-D-thiogalactopyranoside (IPTG) was used at a final concentration of 0.2 or 1 mM.

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TABLE 1. Bacterial strains and plasmids used in this study

| Strains, vector, or plasmid | Relevant characteristic(s) | Source or reference |
|---|---|---|
| Strains | | |
| *V. vulnificus* | | |
| C7184/OP | Wild type (clinical isolate) | 73 |
| C7184DΩ | C7184 with disrupted *pilD* | This laboratory |
| C7184AΩ | C7184 with disrupted *pilA* | This study |
| MO6-24/OP | Wild type (clinical isolate) | 34 |
| PAC1 | Wild type (environmental isolate) | M. Coyle |
| *P. aeruginosa* | | |
| PAK-NP | Nonpiliated mutant | 46 |
| PAK-NP(pRP383) | Nonpiliated mutant expressing PilA from *V. vulnificus* | This study |
| *E. coli* | | |
| DH5α | *supE44lacU169*(Δ*lacZ*ΔM15)*hsdR17 recA lendA1gyrA96 thi-1 relA1* | BRL |
| SM10-λpir | *thi thr leu tonAlacY supE recA*[RP4-2-Tc::Mu] λpir*R6K* $Km^r$ | 51 |
| BL21 | $F^-$ *ompT hsd*$S_B$ ($r_B^-$ $m_B^-$)*gal dcm* | 61, 62 |
| pFLAG-MAC | $Ap^r$ FLAG fusion cloning vector | Sigma |
| pFLAG-ATS | $Ap^r$ FLAG fusion cloning vector | Sigma |
| Cloning vectors | | |
| pZErO-2 | $Kan^r$ Zero background cloning vector | Invitrogen |
| pMMB67EH.cam | $Cm^r$ $Ap^r$ broad-host-range cloning vector, *lacI*$^q$/*tac* promoter | 17 |
| pBluescript II $KS^+$ | $Ap^r$ phagemid cloning vector | Stratagene |
| pEP185.2 | $Cm^r$ suicide vector | 22 |
| Recombinant plasmids | | |
| pRP-P38 | 9- to 10-kb PstI fragment from C7184 carrying the *pilABCD* cluster cloned into pZErO-2 | This study |
| pRP102 | 2.04-kb PCR-generated fragment encompassing *pilA* cloned into $pKS^+$ | This study |
| pRP102Ω | 2.04-kb PCR-generated fragment encompassing *pilA,* disrupted with the 2.0-kb Ω interposon, cloned into pEP185.2 | This study |
| pRP383 | 624-bp XhoI-HindIII fragment encompassing *pilA* cloned into pMMB67EH.cam | This study |
| pRPA110 | PCR-generated *pilA* cloned into pFLAG-MAC | This study |
| pRPA111 | PCR-generated *pilA* cloned into pFLAG-ATS | This study |

**DNA manipulations.** *V. vulnificus* chromosomal DNA was extracted following procedures described for *P. aeruginosa* (56). Standard techniques were used for plasmid extractions, transformations, ligations, and DNA electrophoresis (47). Probes used for Southern blot analysis 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-methoxyspiro {1,2-dioxetane-3,2′-(5-chloro)tricyclo[3,3,1.1$^{3,7}$] decan-4}-yl) phenyl phosphate (CSPD) (Genius System nonradioactive detection kit, version 2.0; Boehringer Mannheim, Indianapolis, Ind.).

**Nucleotide sequence determination.** Double-stranded DNA sequencing was performed with the automated ABI Prism 310 sequencer (ABI Prism, PE Applied Biosystems, Foster City, Calif.) using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction kit and analyzed with Sequencher software (Gene Codes Corporation, Ann Arbor, Mich.). Homology searches were performed with BLAST (1).

**Construction of a *V. vulnificus pilA* mutant strain.** The genetic organization of *pilABCD* and the relevant restriction sites used for the construction of the plasmid pRP383 are shown in Fig. 1A. A *pilA* mutant strain was created by insertion of the Ω interposon (42). Initially, two separate PCR products encompassing *pilA* and with an overlapping SmaI site were combined into one longer product, which was cloned into pBluescript II $KS^+$ to create a plasmid construct carrying *pilA* with a unique SmaI restriction site. Primers used for all PCRs are listed in Table 2. In the primary PCRs, primers VvpACl-1A (SalI site underlined), located 734 bp upstream of the start site of *pilA*, and VvpACl-2 (SmaI site underlined), located 154 bp downstream of the start site of *pilA*, were used to amplify a 902-bp fragment. Similarly, primers VvpACl-3 (SmaI site underlined), located 124 bp downstream of the start site of *pilA*, plus VvpACl-4 (XbaI site underlined), located 1,192 bp downstream of the start site of *pilA*, were used to generate a 1,142-bp PCR fragment. Fragments were gel purified with a GenElute Agarose spin column (Sigma, St. Louis, Mo.) and used as a template in a secondary PCR to generate a single 2-kb fragment carrying *pilA* with a unique SmaI restriction site, using the two primers VvpACl-1A andVvpACl-4. This PCR product was then cloned into pBluescript II $KS^+$, creating plasmid pRP102. The insert from plasmid pRP102 was subsequently transferred to the suicide vector pEP185.2 (22) and disrupted with the 2.0-kb Ω interposon encoding streptomycin and spectinomycin resistance flanked by transcription and translation termination signals at both ends (42), creating plasmid pRP102Ω. The plasmid pRP102Ω was transformed into *E. coli* SM10-λpir and introduced into wild-type C7184 by conjugation. Double recombinants were selected for resistance to streptomycin and spectinomycin and sensitivity to chloramphenicol. A double crossover was confirmed by Southern blot analysis. One isolate, C7184AΩ, was chosen for further study. The orientation of the Ω fragment was determined by restriction analysis as well as sequencing of the plasmid pRP102Ω.

**Complementation of the *V. vulnificus pilA* mutant strain.** The 624-bp XhoI-HindIII fragment encompassing *pilA* was initially cloned into pBluescript II $KS^+$ and subsequently subcloned into pMMB67EH.cam immediately downstream of the inducible *tac* promoter, generating pRP383. This plasmid (in *E. coli* DH5α) was then conjugated into C7184AΩ by triparental mating with *E. coli* DH5α(pRK2013) as the helper strain (8), resulting in C7184AΩ(pRP383). Similarly, pRP383 was conjugated into C7184DΩ, and the vector pMMB67EH.cam was conjugated into C7184AΩ.

**RNA purification and RT-PCR analysis.** Total RNA was purified from mid-log-phase cultures (optical density of 600 nm [$OD_{600}$] = 1.0) of strains C7184 (wild-type) and C7184AΩ (*pilA* mutant) grown in LB broth at 30°C with the appropriate antibiotics, using TRI Reagent and following the manufacturer's protocol (Molecular Research Center, Inc., Cincinnati, Ohio). RNA was treated with amplification-grade DNase 1 (Invitrogen Corporation, Carlsbad, Calif.) to eliminate residual genomic DNA. The purified RNA was used to generate cDNA, using primer VvpilDRT complementary to *pilD*, and Superscript II RNase $H^-$ reverse transcriptase (Invitrogen Corporation), according to the manufacturer's protocol. A control reverse transcription (RT) reaction was included in which the addition of Superscript II RNase $H^-$ reverse transcriptase was omitted. The cDNA obtained from the RT reaction was analyzed by PCR for the presence of transcripts from *pilA*, *pilB*, *pilC*, and *pilD* according to the manufacturer's protocol (Invitrogen Corporation). The following primers (listed in Table 2) were used in the PCRs: VvpAF32 and VvpAR2 (*pilA*), VvpBF11 and VvpBR7 (*pilB*), VvpilCF2 and VvpilCR2 (*pilC*), and VvpilDF1 and VvpilDR1 (*pilD*).

**Preparation of antiserum.** Recombinant PilA protein was prepared with the *E. coli* FLAG expression system (Sigma, St. Louis, Mo.). A DNA fragment consist-

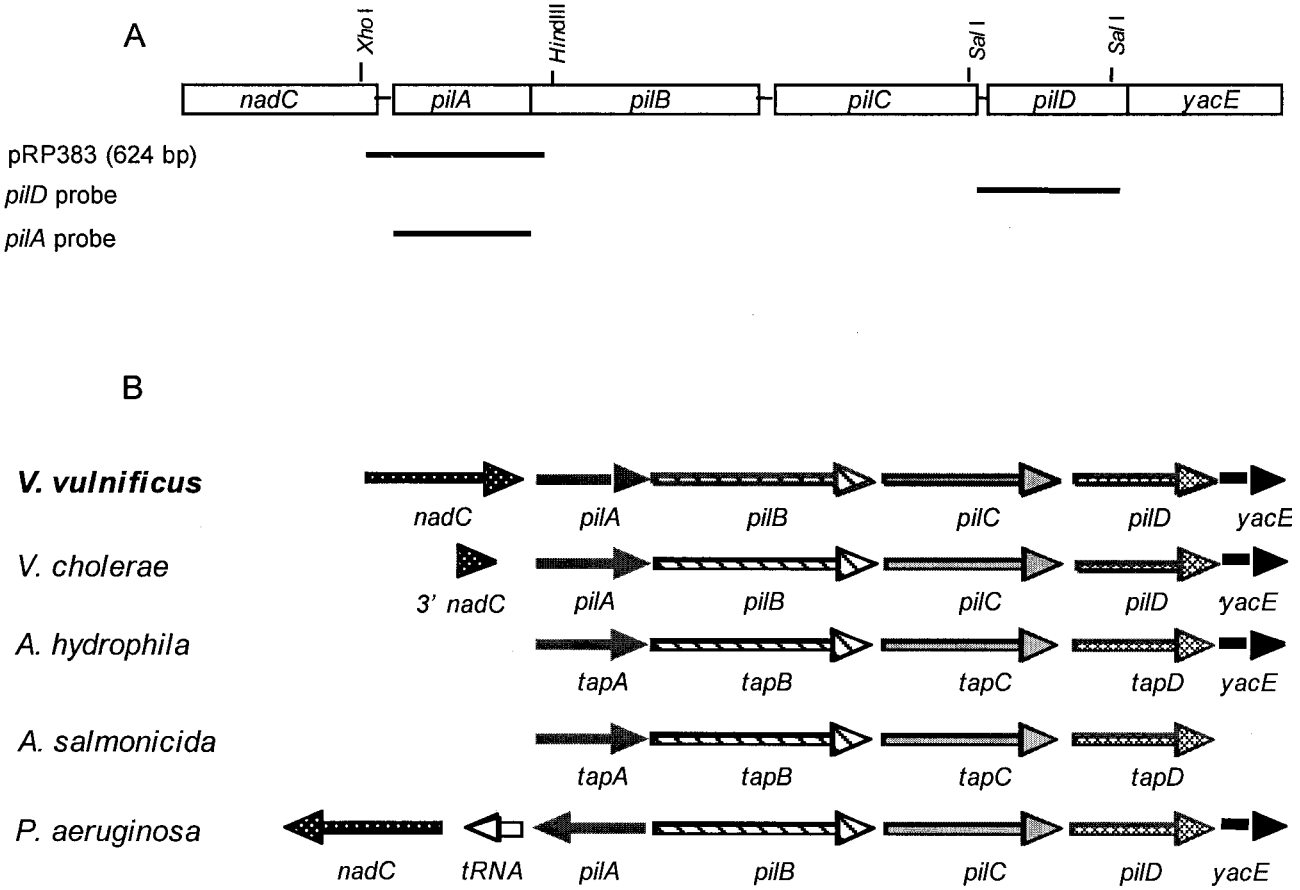


FIG. 1. Genetic organization of the type IV pilus biogenesis gene cluster of *V. vulnificus.* (A) Relevant restriction sites used for cloning constructs and probes. (B) Comparison of the type IV pilus biogenesis *pil* genes of *V. vulnificus* with those of *V. cholerae*, *A. hydrophila*, *A. salmonicida*, and *P. aeruginosa*.

ing of the *pilA* sequence starting immediately downstream from the predicted consensus cleavage site at Phe + 1 was generated by PCR using the primers VvpA-N-pFLAG (with an engineered TthIII l site, underlined) and VvpA-C-pFLAG− (with an engineered EcoRI site, underlined) (Table 2). This fragment was cloned into the amino-terminal portions of both the pFLAG-MAC and the pFLAG-ATS expression vectors with the marker peptide, N-AspTyr-LysAspAspAspAspLys, generating plasmids pRPA110 (using the pFLAG-MAC vector) and pRPA11 (using the pFLAG-ATS vector). The plasmids pRPA110 and pRPA11 were transformed into *E. coli* BL21, and overnight cultures were grown in LB broth with ampicillin and 0.4% glucose at 30°C. Cultures were

TABLE 2. Oligonucleotides used in this study

| Oligonucleotide | Oligonucleotide sequence (5′→3′)[a] | Use |
|---|---|---|
| VvpACl-1A | CTTTCTGGTTGGTCGACCATCACCGC | *pilA* mutant construction |
| VvpACl-2 | CTGTTGCTGATGCCCGGGCAACTTC | *pilA* mutant construction |
| VvpACl-3A | AAACTGAAGTTGCCCGGGCATCAGC | *pilA* mutant construction |
| VvpACl-4 | CGAGCTGTCTAGAAGTCGCAACAC | *pilA* mutant construction |
| VvpA-N-pFLAG | CCAAGAAACAACAACAAGACAAAGTCACCTTGATTG | Recombinant PilA |
| VvpA-C-pFLAG | TTGGCCTGACGCAGAATTCTGGAGAGATTC | Recombinant PilA |
| VvpAF3 | GGTGTGATTTCCACTCATC | *pilA* probe |
| VvpBR6 | CTCTCGACACGCTTGTTC | *pilA* probe |
| VvpilDRT | TACCTGACGAGCGACAATG | RT of *pilD* |
| VvpAF32 | TGGCTGCTGTTGCTATTC | PCR of *pilA* |
| VvpAR2 | GGTCCACCACTAGTACCAAC | PCR of *pilA* |
| VvpBF11 | CTG GTT TAA AAG AGA TTC AC | PCR of *pilB* |
| VvpBR7 | TGC TCG AGC TGT CGA GAA GTC | PCR of *pilB* |
| VvpilCF2 | CCAGTGTGTCAGTTTTCACGAGG | PCR of *pilC* |
| VvpilCR2 | TCGCCGCTTTAGATAAGACCCC | PCR of *pilC* |
| VvpilDF1 | TGAATACAACATCACACCACCGAC | PCR of *pilD* |
| VvpilDR1 | CCTTCTTTCCCTGTCAGCAATCTG | PCR of *pilD* |

[a] Underlined nucleotides represent engineered restriction enzyme sites.

diluted 1:100 in the same medium, induced with IPTG after reaching an $OD_{600}$ of 0.2, and allowed to incubate with shaking for an additional 2 h after induction. Analytical fractionation of the PilA fusion protein indicated that the majority of PilA was associated with the whole-cell fraction, specifically the cell membrane material, when expressed from both pRPA110 and pRPA111. The cell membrane material was solubilized with 0.5% Triton X-100 and the FLAG-labeled PilA purified by immunoaffinity chromatography with the anti-FLAG M2 affinity gel. The PilA-FLAG fusion protein was eluted from the affinity column by competition with FLAG peptide, using 0.1 M glycine, by following the manufacturer's protocol (*E. coli* FLAG expression system; Sigma). The purified PilA-FLAG fusion protein was used to raise polyclonal antiserum in two New Zealand White rabbits (R & R Rabbitry Research Development, Stanwood, Wash.). The rabbits were initially immunized with 0.96 mg of purified PilA-FLAG fusion protein in Freund's complete adjuvant. Five booster injections (0.5 mg of fusion protein in Freund's incomplete adjuvant/booster) were administered at 3-week intervals over a 4-month period. Test bleeds were collected at 55 and 26 days, and 1% bleeds were collected at 97 and 118 days. Serum from the final bleed collected at day 139 was used in this study. The antiserum was adsorbed with an acetone powder preparation (13) of C7184AΩ(pMMB67EH.cam) and tested for PilA specificity.

**Western blot analysis.** Whole-cell lysates of overnight cultures of *V. vulnificus* were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 16 or 18.5% acrylamide gels (26). Following electrophoretic separation, the proteins were transferred to nitrocellulose membranes (65), incubated with polyclonal anti-PilA antiserum, and analyzed with the Renaissance Western Blot Chemiluminescence Reagent *Plus* enhanced luminol kit (NEN Life Science Products, Boston, Mass.).

**Biofilm formation assay.** Biofilm formation was quantified with borosilicate glass tubes (13 by 100 mm), as well as 96-well polyvinyl chloride (Falcon 3911 Microtest III) and polystyrene (Costar) microtiter plates at 25 and 30°C. The assay was conducted by a previously published protocol (39). Briefly, strains to be tested were grown overnight in modified TCG broth with 5-μg/ml chloramphenicol (for strains with the plasmid pMMB67.EH) and 1 mM IPTG (for strains with the plasmid pRP383) at 30°C with shaking. The following day, 200 μl of a 1:100 dilution of each culture was transferred into the appropriate tubes or microtiter plates and incubated statically for 24 h. After incubation, the wells were washed three times with 1× phosphate-buffered saline (PBS), stained with 1% crystal violet for 15 min, and washed three times with PBS. The biofilms were solubilized in 95% ethyl alcohol, and the absorbance was measured at 595 nm. Duplicate assays were performed with 24 replicates of each culture within each assay.

**Tissue culture adherence assays.** Quantitative adherence assays were performed by two methods with human epidermoid carcinoma (HEp-2) cells. In the first method, HEp-2 cells were seeded at a concentration of $2 \times 10^5$and grown overnight in minimal essential medium (MEM) with Earle's salts and 10% fetal bovine serum, on sterile tissue culture-treated coverslips (Thermonox; Nunc no. 174950) in 24-well microtiter plates at 37°C with 5% $CO_2$. The cells were prepared for the assay by washing twice with Hanks' balanced salt solution and incubated for 1 h with 1 ml of serum-free MEM with Earle's salts. Duplicate sets of *V. vulnificus* strains were grown overnight in modified TCG broth with 5 μg of chloramphenicol and 0.2 or 1 mM IPTG per ml (for strains with pRP383) at 30°C with gentle agitation. One set of cultures was radiolabeled by adding 10 μCi of [methyl-$^3$H]thymidine (87.4 mci/mmol) per ml to the broth before overnight incubation. Labeled and unlabeled overnight cultures were adjusted to the same $OD_{600}$, harvested by centrifugation at 6,000 × *g* for 1 min, washed three times in MEM without serum, and resuspended in the same medium. The number of CFU per milliliter was calculated by plating serial 10-fold dilutions of the unlabeled bacteria. The efficiency of labeling for each of the radiolabeled strains was calculated as the number of CFU per cpm (79). Six-cell monolayers were inoculated for each strain tested with ~$8 \times 10^6$ to $9 \times 10^6$ bacteria (multiplicity of infection between 30 and 40), centrifuged at 670 × *g* for 10 min at 10°C, and incubated at 37°C in 5% $CO_2$ for 30 min. Four uninoculated coverslips with HEp-2 cells only were included to determine background cpm. After incubation, the monolayers were washed four times with Hanks' buffered salt solution to remove nonadherent bacteria, and the coverslips were transferred to Ecolume scintillation fluid (ICN Biomedicals (Costa Mesa, Calif.). The number of cpm per coverslip was assessed with a Beckman LS 3801 scintillation counter. The number of bacteria per monolayer was calculated by multiplying the cpm per coverslip by the efficiency of cell labeling.

In the second method, HEp-2 cells were seeded in the slide chambers at a concentration of $10^4$ and grown overnight at 37°C in 5% $CO_2$ (Lab-Tek II chamber slide system; Nalgene, Nunc International, Naperville, Ill.). *V. vulnificus* cultures were grown overnight in modified TCG medium (5-μg/ml chloramphenicol and 0.2 mM IPTG in the strain with pRP383) at 30°C with gentle agitation. After measuring the $OD_{600}$ to estimate the bacterial cell concentration based on previous growth curves, cultures were centrifuged at 6,000 × *g* for 1 min, washed twice in MEM without serum, and diluted to a concentration of $2 \times 10^6$ CFU/ml ($1 \times 10^6$/0.5-ml inoculum). The cell monolayers were prepared for the assay by washing twice with Hanks' balanced salt solution and then were inoculated with 0.5 ml of a bacterial suspension in serum-free MEM (multiplicity of infection of ca. 100) and incubated at 37°C in 5% $CO_2$ for 30 min. Nonadherent bacteria were removed by four washes with Hanks' balanced salt solution. The monolayers were fixed with methanol-acetic acid (3:1) for 5 min, stained with May-Grünwald stain (5 min) and Giemsa stain (10 min), and mounted for evaluation by light microscopy (23).

**Animal studies.** Fifteen- to twenty-gram, female, Swiss Webster mice (Animal Technologies, Ltd., Kent, Wash.) were used in all experiments. Mice were administered intraperitoneal injections of 5 mg of iron dextran 2 h prior to challenge (72). Overnight cultures of bacteria grown in BHI broth at 37°C, with chloramphenicol and IPTG where appropriate, were pelleted, washed once, and diluted to the appropriate concentrations. The mice ($n = 5$ for each dilution) received intraperitoneal inoculations of 10-fold dilutions of the bacteria at concentrations between $5 \times 10^2$ and $5 \times 10^6$ CFU/ml. Chloramphenicol succinate (100 mg) was administered 2 h after the bacterial challenge, to maintain antibiotic selection for the plasmid (78). The experiment was terminated after 26 h, and the 50% lethal dose ($LD_{50}$) was calculated by the method of Reed and Muench (44).

**Southern blot analysis of *V. vulnificus* isolates for the presence of *pilA* and *pilD*.** Strains of *V. vulnificus* were obtained from the Food and Drug Administration (FDA), Dauphin Island, Ala. Chromosomal DNA was digested with PstI and HindIII and analyzed by Southern blot hybridization under high-stringency conditions by standard techniques (47). The *pilA* probe was prepared from a 562-bp fragment that was generated by PCR with the primers VvpAF3 and VvpBR6 encompassing the *pilA* gene, while the *pilD* probe used was prepared from a 1-kb SalI fragment encompassing the *pilD* gene (Table 2 and Fig. 1A). Probes were labeled and DNA was detected as previously described.

**Transmission electron microscopy.** Bacterial cells grown overnight in modified TCG medium at 30°C, were suspended in Karnovsky's fixative (2% freshly depolymerized paraformadehyde plus 2.5% glutaraldehyde in 0.2 M cacodylate buffer) for 1 h at room temperature, centrifuged at 2,000 × *g* for 3 min, and washed once with PBS. Cells were negatively stained with 2% uranyl acetate on Butyar-coated grids and observed with a Phillips CM100 transmission electron microscope at 60 kV.

**Statistical analysis of adherence and biofilm assay results.** Adherence was expressed as the mean number of bound bacteria per coverslip ± the standard deviation or the mean number of adherent bacteria per epithelial cell ± the standard deviation. The significance of the difference was calculated by one-way analysis of variance (ANOVA) and Fisher's protected least significant difference (PLSD) test (53). Differences were accepted as statistically significant if $P < 0.05$. The significance of difference for the absorbance values in the biofilm formation assay was similarly calculated.

**Nucleotide sequence accession number.** The nucleotide sequences of the C7184 *nadC*, *pilA*, and *pilB* genes have been deposited in the GenBank/EMBL/DDBJ data libraries under accession no. AF439854.

## RESULTS

**Cloning and sequence analysis of *V. vulnificus pilA* and *pilB*.** The organization and function of genes required for type IV pilus biogenesis in gram-negative pathogens are highly conserved. In several organisms, such as *V. cholerae*, *P. aeruginosa*, *A. hydrophila*, and *A. salmonicida*, four of the approximately 35 genes required for type IV pilus biogenesis, *pilABCD*, are arranged in a cluster (Fig. 1B) (10, 30, 36, 41, 67). In a previous study, we characterized the *pilC* (*vvpC*) and *pilD* (*vvpD*) genes from *V. vulnificus* (40). To determine if *V. vulnificus* encodes additional pilus biogenesis genes clustered in an arrangement similar to that seen in related gram-negative pathogens, the region upstream of *pilCD* was cloned. Chromosomal DNA from *V. vulnificus* C7184 was digested with several restriction enzymes and analyzed by Southern blot hybridization under high-stringency conditions for the presence of the pilus biogenesis genes, using an oligonucleotide probe generated from


FIG. 2. (A) Comparison of the amino acid sequence of *V. vulnificus* PilA with the homologous type IV pilins of *V. cholerae* (Vc PilA), *A. hydrophila* (Ah TapA), *P. aeruginosa* (Pa PilA), and *V. cholerae* (Vc MshA). Conserved residues around the cleavage site are shaded. The remainder of the sequence is less homologous. The inverted triangle indicates the consensus cleavage site for group A, type IV pilins. (B) Comparison of the amino acid sequence of *V. vulnificus* PilB with homologs from *V. cholerae* (Vc PilB), *A. hydrophila* (Ah TapB), and *P. aeruginosa* (Pa PilB). The location of the Walker box motif is shaded. Both alignments were generated by using the Pileup program of the Genetics Computer Group (Madison, Wis.).

the 1-kb SalI fragment encompassing the *pilD* gene (Fig. 1A). Size-fractionated DNA from PstI digests indicated the presence of *pilD* on a 9- to 10-kb fragment. DNA fragments in the desired size range were gel purified (GenElute columns; Sigma) and cloned into pZEro-2 (Invitrogen, Carlsbad, Calif.).

Over 400 recombinant clones carrying the size-fractionated DNA were screened for the presence of the pilus biogenesis genes by Southern blot hybridization with the same *pilD* oligonucleotide probe. One plasmid carrying a 9-kb PstI fragment was probe positive for the presence of *pilD* and was designated pRP-P38. Since *pilC* and *pilD* had been previously sequenced, the region upstream from *pilC* was sequenced, beginning with reverse primers designed from the 5′ end of *pilC*.

Sequencing upstream of the known *pilC* sequence revealed the presence of three complete open reading frames (ORFs). Immediately upstream of *pilC* was a 1,686-bp ORF encoding a protein of 562 amino acids. This protein, designated PilB, showed 69% identity and 79% similarity to PilB from *V. cholerae*, 49% identity and 66% similarity to TapB from both *A. hydrophila* and *A. salmonicida*, and 47% identity and 63% similarity to PilB from *P. aeruginosa*, using BLAST (1). These proteins are required for type IV pilus biogenesis and contain a nucleotide-binding region (Walker box A, consensus sequence GXXGXGKT) characteristic of ABC transporter proteins that are involved in transport of a wide variety of substrates in both directions across the cell membranes of several eukaryotic and prokaryotic organisms (66) (Fig. 2B). The second ORF of 441 bp encodes a protein of 147 amino acids. Comparison of this ORF to the GenBank database by BLAST showed considerable homology within the N-terminal region of about 30 amino acids to type IV pilin precursor proteins of *V. cholerae*, *A. hydrophila*, and *P. aeruginosa* and several other gram-negative pathogens. The N terminus of the polypeptide is characteristically dominated by hydrophobic residues and reveals the presence of the conserved consensus cleavage site with the FTLIE motif that forms the first five amino acids of the mature protein of the type IV prepilins (55) (Fig. 2A). This gene was designated *pilA* following previously established nomenclature for genes encoding the pilin precursor protein in the type IV pilus biogenesis gene cluster. In *V. vulnificus*, the

invariant glycine residue is at position 12 and the phenylalanine at position 13 of the prepilin, suggesting a leader peptide 12 amino acids long, which closely resembles the 11-amino-acid type IV leader sequence of the *V. cholerae* PilA but is a slight variation from the more typical 5- to 7-amino-acid leader sequences of the type IV, group A pilin family. The remaining sequence is less conserved and contains the region that typically encodes the antigenic epitopes of type IV pili (57). The carboxy-terminal domain contains the characteristic pair of cysteine residues that are known to be involved in formation of a disulfide bond that may be important for pilin subunit interactions (14). The *pilA* sequence also indicates the presence of a potential $\sigma^{54}$-dependent RpoN promoter binding site, $_{-247}$GGATTTATCGATGC$_{-234}$, upstream of the predicted start site of the pilin sequence and a ribosomal binding site, $_{-13}$AAAGGA$_{-8}$, upstream of the ATG initiation codon.

The third ORF, of 885 bp, upstream from *pilA*, encodes a protein of 295 amino acids. This protein, with homology to quinolinate phosphoribosyltransferases, was designated NadC because of its homology to *V. cholerae* NadC, a nicotinate-nucleotide pyrophosphorylase. It shares 80% identity and 89% similarity to the *V. cholerae* NadC and is located upstream of the *pil* gene cluster, as it is in *V. cholerae* and *E. coli* (10, 67).

The *pil* gene cluster from *V. vulnificus* C7184 also appears to be highly conserved when compared to those from the two recently sequenced *V. vulnificus* strains CMCP6 and YJ016 (GenBank accession no. AEO16795 and BA000037). *V. vulnificus* C7184 PilA shares 79% identity to PilA from strain CMCP6, and 41% identity to PilA from YJ016. The more highly conserved type IV assembly protein, PilB from strain C7184, shares 94 and 93% identity to PilB from CMCP6 and YJ016, respectively. Similarly, C7184 PilC shares 91% identity with PilC from both CMCP6 and YJ016, and the type IV prepilin peptidase PilD shares 91% and 90% identity, respectively. The comparison to the two sequenced strains shows that the *pil* cluster is located on chromosome I of *V. vulnificus.*

**Expression of PilA in *V. vulnificus*.** A mutation in *pilA* was constructed by insertion of the Ω fragment as described in Materials and Methods to create strain C7184AΩ. Strain C7184AΩ was initially compared to the wild-type strain, C7184, for differences in growth in LB broth and BHI broth at both 30 and 37°C. Growth of C7184AΩ was comparable to that of the wild-type strain, C7184, under the conditions tested, verifying that the mutation in *pilA* did not affect its rate of growth.

Expression of PilA was then compared in the *V. vulnificus* wild type, C7184(pMMB67EH.cam), and the *pilA* mutant, C7184AΩ (pMMB67EH.cam), strains by Western blot analysis. Cultures were grown in LB, BHI, Trypticase soy, Marine broth, and TCG broth (6) and on agar medium with the same formulations, with the pH adjusted to 5.5, 6.5, or 7.2. The incubation temperature was also varied, with the cultures grown at 22, 30, or 37°C. Whole-cell lysates were examined by Western blot analysis with polyclonal anti-PilA antiserum (see Materials and Methods). Expression of PilA was optimal in cultures grown in TCG broth at 30 and 37°C. The results show that a protein of ~14 kDa reacted with the PilA antiserum in whole-cell lysates of the wild-type strain but not in the *pilA* mutant strain (Fig. 3, lanes 1 and 2). Complementation of the *pilA* mutation in C7184AΩ with pRP383 (see Materials and Methods) restored PilA expression when induced with IPTG (Fig. 3, lane 3). The plasmid pRP383 was also conjugated into the *pilD* mutant strain, C7184DΩ, and induced with IPTG. Whole-cell lysates of C7184DΩ(pRP383), show an immunoreactive band of ~15.5 kDa, which corresponds to the unprocessed form of PilA and includes the 12-amino-acid leader sequence (Fig. 3, lane 4). This demonstrates that the PilA precursor is a substrate of PilD. Cell fractionation of the wild type, *pilA* mutant, *pilD* mutant, and the complemented *pilA* mutant strains indicates that both the processed and the unprocessed forms of PilA remain associated with the membrane fraction (data not shown) (56).

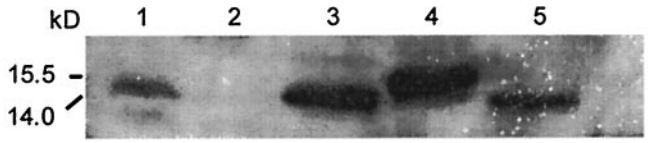


FIG. 3. Expression of PilA in *V. vulnificus* strains grown in TCG medium at 30°C. Whole-cell lysates were analyzed by Western blotting with anti-PilA antiserum. Lane 1, C7184 (wild-type); lane 2, C7184AΩ (*pilA* mutant); lane 3, C7184AΩ (*pilA*$^+$); lane 4, C7184DΩ (*pilA*$^+$); lane 5, PilA (purified).

**RT-PCR analysis of the *pilABCD* gene cluster.** The wild-type and *pilA* mutant strains were compared by RT-PCR to verify that the other pilus biogenesis genes downstream of *pilA* are still transcribed in the *pilA* mutant strain and that the mutation is not polar. Total RNA extracted from the wild-type strain, C7184, and the *pilA* mutant strain, C7184AΩ, was subjected to RT-PCR analysis as described in Materials and Methods. The cDNA generated by primer VvpilDRT was examined for the presence of transcripts from *pilA*, *pilB*, *pilC*, and *pilD* by PCR, using the specific primers internal to each of the genes (Table 2). The analysis shows the presence of PCR fragments of the expected size from the wild-type strain resulting from transcription of *pilA* (217 bp), *pilB* (432 bp), *pilC* (666 bp), and *pilD* (471 bp) (Fig. 4, lanes 1 to 4) from the cDNA generated with the primer VvpilDRT, which is complementary to *pilD*. Lanes 5 to 8 of Fig. 4 show PCR products from transcription of the same genes from the *pilA* mutant strain. The absence of a PCR product in lane 5 verifies that the mutation in *pilA* resulted in loss of transcription. Lanes 6, 7, and 8 show PCR products that are comparable to those in the wild type, confirming that *pilB*, *pilC*, and *pilD* are still transcribed in the *pilA* mutant strain, under the conditions tested. The negative control RT reactions corresponding to lanes 1 to 8 are shown in lanes 9 to 16. The detection of transcripts from *pilA*, *pilB*, *pilC*, and *pilD* from the cDNA generated with the primer complementary to *pilD*, confirms that *pilABCD* are organized in an operon similar to that suggested for *V. cholerae* (10), but which is different from what was demonstrated for *P. aeruginosa pilBCD* transcription (25). A previous study has shown that an insertion of the Ω fragment in one orientation can be nonpolar, only affecting the gene that it is inserted into and not the downstream genes (35). This explains why inserting the Ω fragment in *pilA* has no effect on transcription of *pilBCD*.

**Expression of pili on the surface of *V. vulnificus*.** In a previous study, we demonstrated that a mutation in *vvpD* (now *pilD*), the gene encoding the type IV leader peptidase/*N*-meth-

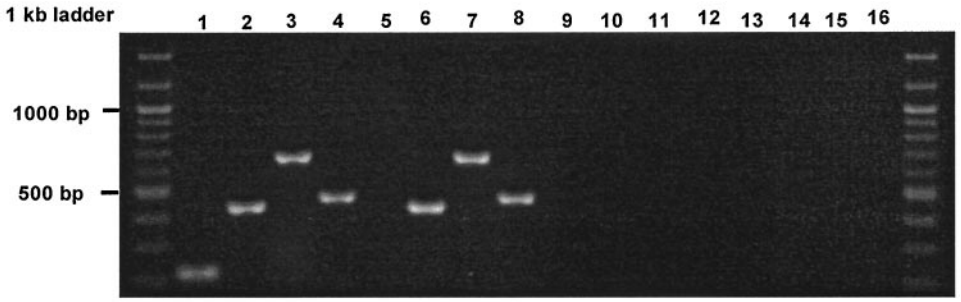


FIG. 4. Detection of *pilA*, *pilB*, *pilC*, and *pilD* transcripts by RT-PCR in *V. vulnificus* C7184 (wild type) (lanes 1 to 4) and C7184AΩ (*pilA* mutant) strains (lanes 5 to 8). The corresponding control RT reactions in which the addition of Superscript II RNase $H^-$ reverse transcriptase was omitted are shown in lanes 9 to 16.

yltransferase (type IV prepilin peptidase), results in absence of pili on the surface of *V. vulnificus* (40). These pili must therefore be processed by PilD and thus belong to the type IV pilus group. However, examination of C7184AΩ by transmission electron microscopy demonstrates that pili are still present on the surface of the *pilA* mutant strain, with no discernible difference in the total number or morphology of the pili as compared to those expressed by the wild-type cells. This suggests that PilD from *V. vulnificus* processes more than one type IV pilin precursor (Fig. 5B). The recently published genome sequences of *V. vulnificus* confirm the presence of at least one other type IV pilus gene cluster (*mshA*) (3, 21), but only a single gene encoding a type IV prepilin peptidase (*pilD*). Pili observed on the surface of IPTG-induced *V. vulnificus* C7184AΩ(pRP383) were also difficult to distinguish from those expressed by the wild type or the *pilA* mutant strain (Fig. 5C). To verify that PilA is competent for pilus formation, plasmid pRP383, carrying the *pilA* gene, was conjugated into *P. aeruginosa* PAK-NP in which PilA has been inactivated by insertion of a tetracycline resistance cassette (46). *V. vulnificus*

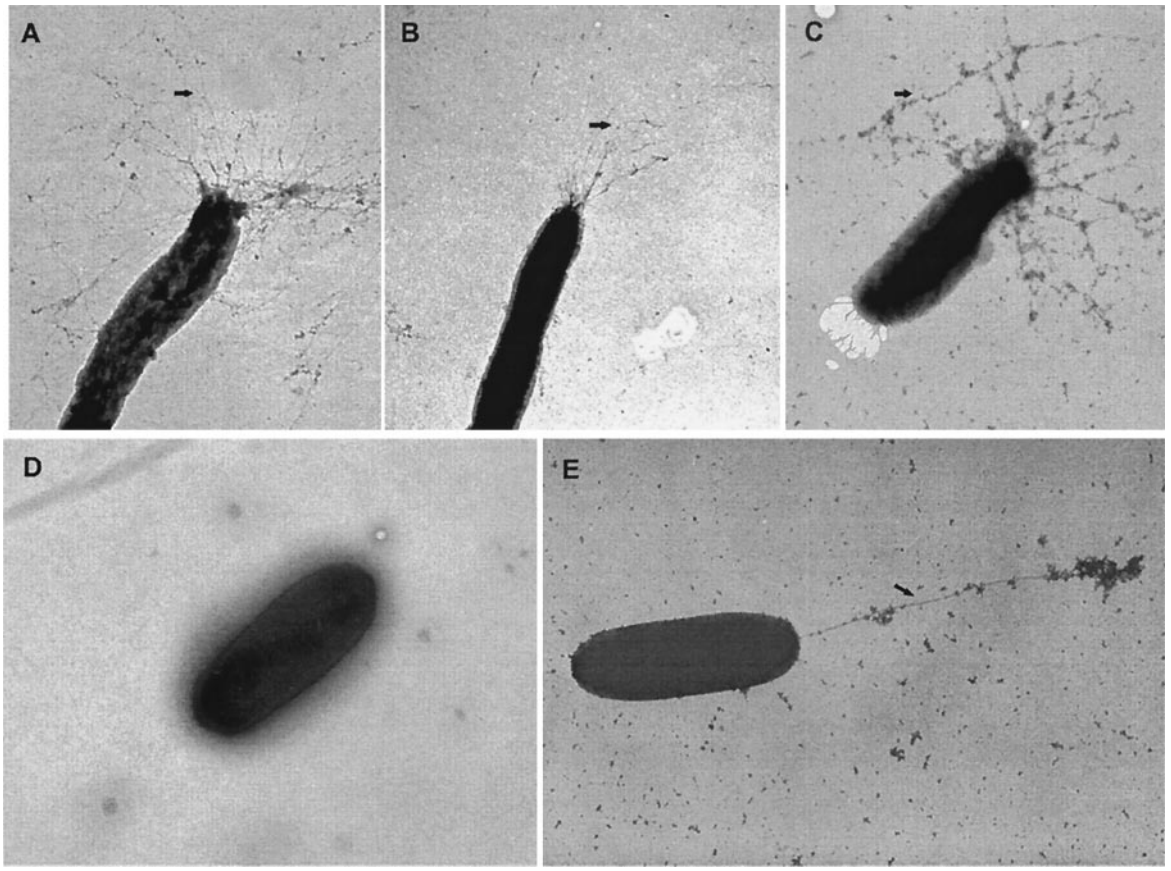


FIG. 5. Transmission electron micrographs of *V. vulnificus* C7184 strains showing surface pili (indicated by arrows). Cells were negatively stained with 2% uranyl acetate on Butyar-coated grids. (A) C7184; (B) C7184AΩ; (C) C7184AΩ(pRP383); (D) *P. aeruginosa* PAK-NP; (E) *P. aeruginosa* PAK-NP(pRP383). Magnification, ×15,500.

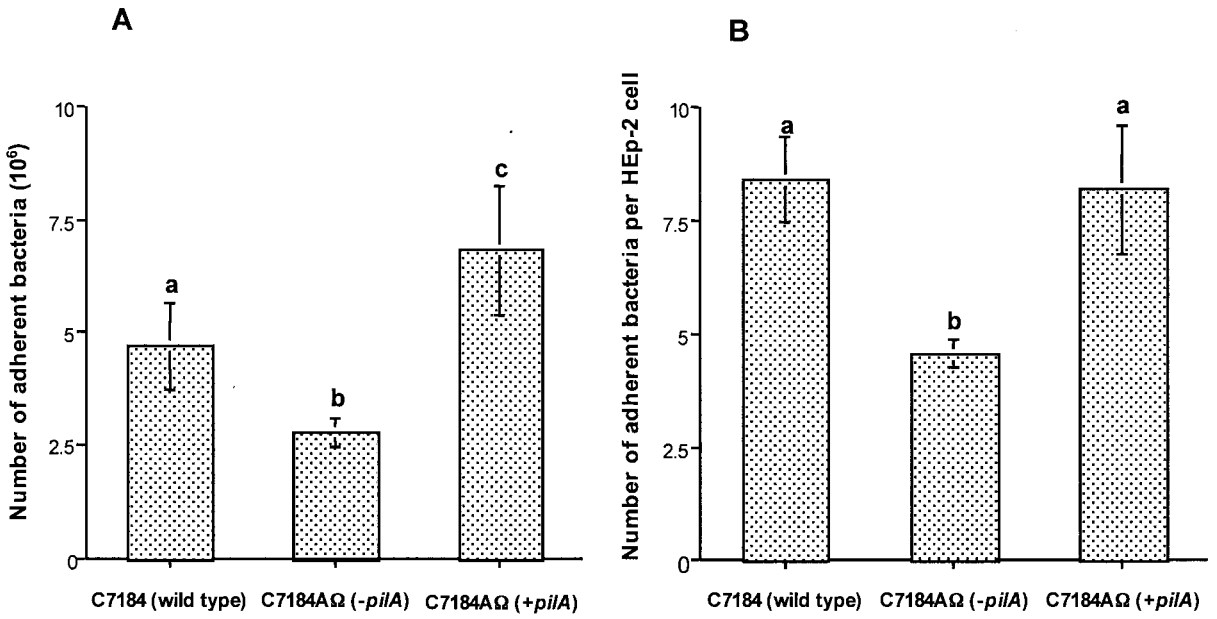


FIG. 6. Comparison of adherence of *V. vulnificus* strains to epithelial cells (HEp-2). (A) Adherence was determined with *V. vulnificus* strains labeled with [methyl-$^3$H]thymidine. (B) One hundred epithelial cells were counted for each strain. Values represent the mean number ± standard deviation of adherent bacteria per coverslip (A) or the number of adherent bacteria per epithelial cell (B). Values denoted by different letters are significantly different when compared by a one-way ANOVA and Fisher's PLSD at $P \leq 0.05$.

PilA is expressed in *P. aeruginosa* PAK-NP, when examined by Western blot analysis. When *P. aeruginosa* PAK-NP(pRP383) cells were examined by transmission electron microscopy, polar pili were easily observed on the surface, confirming that PilA can form a pilus structure (Fig. 5E).

**Adherence of *V. vulnificus* to cultured epithelial cells.** To determine if *V. vulnificus* PilA contributes to the ability of the bacteria to adhere to HEp-2 cells, strains C7184, C7184AΩ, and C7184AΩ(pRP383) were compared by two tissue culture adherence assay methods: one in which the bacteria were radiolabeled and a second in which bacteria were stained before determining adherence, as described in Materials and Methods. For these assays, cultures were grown in modified TCG medium in which PilA expression is highest. EGTA was omitted from the original TCG formulation for cultures used in this assay, since its presence caused cell elongation, chain formation, and premature lysis of cells. In addition the concentration of IPTG was lowered to 0.2 mM (final concentration). The omission of EGTA and the reduced concentration of IPTG from TCG did not affect the expression of PilA, as verified by Western blot analysis (data not shown). Growth and colony morphology were also comparable for C7184, C7184AΩ, and C7184AΩ(pRP383). The *pilA* mutant (C7184AΩ) was found to be impaired in its ability to adhere to HEp-2 cells, when examined by both the methods of assessing adherence (Fig. 6). When adherence was measured with radiolabeled bacteria as outlined in Materials and Methods, 23.4% of the wild-type (C7184) bacteria remained adherent to the epithelial cells as compared to 13.9% of the bacteria with the *pilA* mutation (C7184AΩ) (significantly different when compared by one-way ANOVA and Fisher's PLSD at $P \leq 0.05$). Complementation of C7184AΩ with *pilA* in *trans* on the plasmid pRP383 restored adherence, with 34% of the bacteria remaining adherent. The higher adherence of the complemented mutant strain may be due to increased expression of PilA from the inducible *tac* promoter. In assays where the adherent bacteria were enumerated after staining, 8.3% of the wild-type bacteria remained adherent as compared to 4.6% of the *pilA* mutant strain (significantly different when compared by one-way ANOVA and Fisher's PLSD at $P \leq 0.05$). Adherence of the complemented *pilA* mutant strain was comparable to that of the wild type strain, with 8.4% of the bacteria remaining adherent. The difference in overall adherence as measured by the two methods may be due to the fact that the radiolabeled method will measure adherent bacteria that are not readily visible microscopically. It should also be pointed out that in a previous study, a mutation in *pilD*, the gene encoding the type IV prepilin peptidase, resulted in a substantial decrease in adherence as compared to the wild-type strain, when ca. 9% of wild-type cells remained adherent to HEp-2 cells as compared to less than 0.5% of the *pilD* mutant cells (39). The difference in adherence between the *pilA* and *pilD* mutants may be due to the fact that no pili are expressed on the surface of the latter mutant.

**Role of *V. vulnificus* PilA in biofilm formation.** Type IV pili from several gram-negative bacteria are essential for the initial attachment and microcolony formation during biofilm development (24, 68). To determine if *V. vulnificus* PilA contributes to biofilm formation, we compared the ability of the wild-type, *pilA* mutant, and the complemented *pilA* mutant strains to form biofilms on borosilicate glass, polyvinyl chloride, and polystyrene. The results show a significant decrease in biofilm formation on borosilicate glass by the *pilA* mutant strain (C7184AΩ) as compared to the wild-type and complemented *pilA* mutant strains (Table 3). A smaller difference in biofilm formation between the wild-type and *pilA* mutant strains was

TABLE 3. Comparison of biofilm formation[a] on borosilicate glass by *V. vulnificus* C7184 (wild type), C7184AΩ (*pilA* mutant), and C7184AΩ (*pilA*)$^+$

| Strain | $A_{595}$[b] |
|---|---|
| C7184 (wild-type) | 4.823 ± 0.10* |
| C7184AΩ (*pilA* mutant) | 3.571 ± 1.15† |
| C7184AΩ (*pilA*)$^+$ | 4.730 ± 0.19* |
| Control (no bacteria) | 0.642 ± 0.38‡ |

[a] Biofilms were stained with 1% crystal violet, solubilized in 95% ethanol, and the $A_{595}$ was measured.
[b] Values represent the mean ± standard deviation of an average of 24 replicate samples. Values with different symbols significantly differ when compared by one-way ANOVA and Fisher's PLSD at $P \leq 0.001$.

noted when polystyrene and polyvinyl chloride surfaces were used as substrates (data not shown).

**Role of *V. vulnificus* PilA in virulence.** To determine if PilA is required for virulence of *V. vulnificus*, the $LD_{50}$ values of the wild-type, the *pilA* mutant, and the complemented *pilA* mutant strains were determined in the iron dextran-treated mouse model (73). Mice ($n = 5$ for each dilution) were inoculated intraperitoneally with 10-fold dilutions of the bacteria at concentrations between $5 \times 10^2$ and $5 \times 10^6$ CFU/ml. As seen in Table 4, the $LD_{50}$ of C7184AΩ is 1 log higher than that of the wild-type strain, C7184. The decrease in virulence was completely restored in the complemented *pilA* mutant strain, C7184AΩ(pRP383), indicating that expression of PilA is required for full virulence of *V. vulnificus* in this model. In the previous study, a mutation in *pilD* resulted in more than a 2-log decrease in the $LD_{50}$ as compared to the level in the wild-type strain (40).

*V. vulnificus* virulence has been associated with the amount and presence of the polysaccharide capsule (33, 74, 76). Loss of capsule in *V. vulnificus* strains results in a translucent phenotype. Colonies of *V. vulnificus* C7184AΩ are opaque and indistinguishable from the wild-type strain. When capsule expression was analyzed in an assay based on hydrophobicity in ammonium sulfate (76), the wild-type strain, C7184, and the *pilA* mutant strain, C7184AΩ, were equally hydrophobic, confirming that the decrease in virulence of the *pilA* mutant strain was not due to reduced capsule expression (data not shown). Previously, we have shown that the nonpiliated *pilD V. vulnificus* mutant is also fully encapsulated (40).

**Analysis of *V. vulnificus* clinical and environmental isolates for the presence of *pilA* and *pilD*.** Twenty-seven *V. vulnificus* isolates, which include 10 clinical isolates, 7 isolates from shellfish (mainly oysters) associated with disease, and 10 environmental (oyster) isolates (Table 5), were compared by Southern blot analysis for the presence of *pilA* and *pilD*. Two clinical isolates, C7184 and MO6-24, and an environmental isolate, PAC1, were used as reference strains. Southern blot hybridization, using the PCR-generated 562-bp *pilA* probe, indicated the presence of *pilA* on an ~6-kb HindIII fragment in all of the isolates, including the reference strains *V. vulnificus* MO6-24 and C7184 (Fig. 7). The environmental isolate, PAC1, differs from all other strains studied, with a 3-kb fragment hybridizing to the *pilA* probe. In a similar analysis of the same isolates, *pilD* was detected on a 1-kb SalI fragment in all strains tested (data not shown).

TABLE 4. Comparison of virulence of *V. vulnificus* strains in mice pretreated with iron dextran

| Strain | $LD_{50}$ ($log_{10}$)[a] |
|---|---|
| C7184 (wild type) | 3.27 |
| C7184AΩ (*pilA* mutant) | 4.36 |
| C7184AΩ (*pilA*$^+$) | 3.54 |

[a] $LD_{50}$S were calculated by the method of Reed and Muench (44).

TABLE 5. *V. vulnificus* isolates used to determine the presence of *pilA* and *pilD* (Fig. 7)[a]

| Strain | Description | Location (state) |
|---|---|---|
| CDC9393-95 | Shellfish isolate associated with illness | TX |
| Oyster isolate 2 | Oyster isolate | FL |
| CDC9356-95 | Shellfish isolate associated with illness | LA |
| Oyster isolate 3 | Oyster isolate | NC |
| CDC9315-95 | Shellfish isolate associated with illness | FL |
| CDC9424-95 | Shellfish isolate associated with illness | FL |
| Oyster isolate 1 | Oyster isolate | FL |
| Oyster isolate 5 | Oyster isolate | LA |
| Oyster isolate 6 | Oyster isolate | LA |
| CDC9029-95 | Clinical isolate | FL |
| Oyster isolate 4 | Oyster isolate | LA |
| CDC9369-95 | Shellfish isolate associated with illness | AL |
| CDC9396-95 | Shellfish isolate associated with illness | TX |
| CDC9047-96 | Clinical isolate | LA |
| CDC9038-96 | Clinical isolate | TX |
| CDC9383-96 | Shellfish isolate associated with illness | LA |
| Oyster isolate 7 | Oyster isolate | LA |
| CDC8355-95 | Shellfish isolate associated with illness | LA |
| CDC9057-96 | Clinical isolate | TX |
| CDC9026-95 | Clinical isolate | FL |
| CDC9345-95 | Clinical isolate | LA |
| CDC9075-96 | Clinical isolate | FL |
| CDC9058-96 | Clinical isolate | LA |
| CDC9049-96 | Clinical isolate | TX |
| CDC9039-96 | Clinical isolate | LA |
| CDC9346-95 | Clinical isolate | FL |
| Oyster isolate 8 | Oyster isolate | FL |

[a] Isolates were obtained from the U.S. Food and Drug Administration, Dauphin Island, Ala.

## DISCUSSION

The identification of factors that contribute to the pathogenesis of *V. vulnificus* has been especially challenging. Several bacterial cell-associated and -secreted proteins have been implicated in the ability of *V. vulnificus* to cause disease, but only the presence and amount of the exopolysaccharide capsule, the presence of a siderophore to acquire iron from transferring, and a functional flagellar biogenesis system have been positively correlated with virulence (28, 52, 74, 76, 77). The precise roles of the many toxins and enzymes that are secreted remain to be defined (7, 64, 72, 75). Disruption of individual genes encoding proteins that have potent in vitro pharmacological activities, including a cytolysin (72), a metalloprotease (50), and an elastolytic protease (16, 27), has shown that none of these are essential virulence determinants, since the mutants are just as pathogenic as the parental strains. In fact, a metalloprotease-deficient mutant was found to be even more virulent than wild-type *V. vulnificus* in orally challenged mice (50).

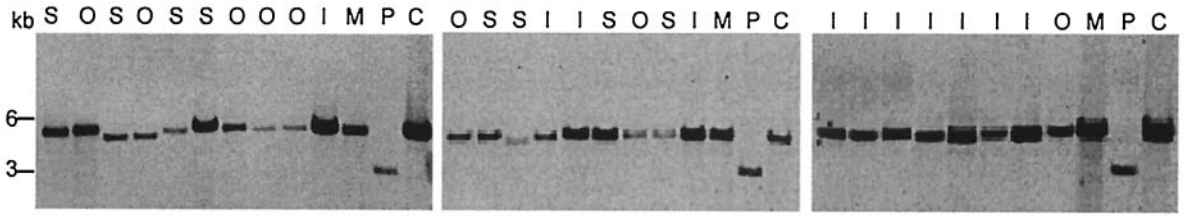


FIG. 7. Southern blot analysis of clinical and environmental isolates of *V. vulnificus*, using a probe encompassing the *pilA* gene (see Materials and Methods). I, clinical isolates; S, shellfish isolates associated with illness; O, oyster isolates not associated with illness. M, strain MO6-24; P, PAC1; C, C7184.

It was noted that lack of metalloprotease expression resulted in an increase in activity of the cytolysin, suggesting that the protease may be responsible for degradation of the cytolysin in the wild-type strain. In a follow-up study, the virulence and cytotoxicity of a *V. vulnificus* double mutant deficient in expression of both the cytolysin and metalloprotease were also found to be comparable to those of the wild-type strain, indicating that neither factor was essential for virulence. However, the increase in the accumulation of the cytolysin in the absence of the metalloprotease may account for the observed increase in intestinal damage in orally challenged mice (7, 75).

There is increasing evidence suggesting that virulence in *V. vulnificus* is multifactorial. Our previous study showed that a *pilD* mutant defective for pilus expression and secretion of proteins (including the cytolysin and metalloprotease) is significantly less virulent (40). A mutation in *pilD* caused a broad pleiotropic defect that prevents expression of pili on the surface and in addition blocks extracellular secretion of several proteins that utilize the type II secretion pathway (40). This resulted in a significant reduction in bacterial cytotoxic activity, adherence to cultured epithelial cells, and virulence in mice pretreated with iron dextran. However, the mutation in *pilD* did not affect expression of the exopolysaccharide capsule, suggesting that in addition to the capsule, a combination of pili and/or multiple secreted proteins also play a significant role in the pathogenesis of this organism. In a recent study which examined the role of a quorum-sensing system in the virulence of *V. vulnificus*, it was noted that a defect in the *luxS* gene, encoding the autoinducer 2 (Al-2) synthase in *V. vulnificus* caused a decrease in protease production, cytotoxicy to HeLa cells, and lethality to mice, but resulted in an increase in hemolysin production, implying that the LuxS quorum-sensing system is likely involved in coordinating the regulation of virulence expression during infection (20). The regulation of expression of a metalloprotease, biofilm formation, and starvation adaptation by SmcR, the *Vibrio harveyi* Lux R homologue in *V. vulnificus*, has also been reported (31). Identification of *toxRS*, which encodes a transcriptional activator in *V. vulnificus* that regulates production of hemolysin and is homologous to the *V. cholerae* transmembrane regulator ($toxRS_{vc}$), also suggests that regulation of virulence in *V. vulnificus* may be controlled by a complex regulatory system (27, 32).

Type IV pili have been shown to be essential for adherence to host cells in *P. aeruginosa* and *Neisseria gonorrhoeae* (11, 19), and their role as important determinants of pathogenicity has been established in *P. aeruginosa* (11), *N. gonorrhoeae* (63), *Neisseria meningitidis* (54), *Moraxella bovis* (45), and *Dichelobacter nodosus* (54). Many proteins required for type IV pilus biogenesis share extensive homology among gram-negative pathogens, and a subset of the genes encoding these proteins are often clustered on the chromosome. Our study shows that *V. vulnificus* PilA has a distinct role in attachment to human epithelial cells. The smaller defect in adherence of the *pilA* mutant strain as compared to the *pilD* mutant strain supports the concept that PilA is one of several proteins processed by PilD that are likely to be involved in adherence of this bacterium to epithelial cells. Expression of PilA in vitro required specific culture conditions, which suggests that this pilin is expressed at low levels under in vitro conditions. However in vivo expression of this pilin may be substantially higher than in vitro expression, since the *pilA* mutant strain was clearly less virulent in the mouse model than the wild-type strain. This verifies that PilA has a definite role in pathogenesis of this organism. This decrease in virulence in the *pilA* mutant strain is independent of the expression or deposition of the exopolysaccharide capsule, the other major cell surface component required for virulence, as there was no difference in the presence or the amount of capsule expressed by the wild-type strain and *pilA* mutant strain. A recent analysis of genes uniquely expressed during human infection with *V. cholerae* suggests that the homologous PilA from *V. cholerae* is expressed during infection and may be involved in colonization of the gastrointestinal tract (12). A more specific role of *V. cholerae* PilA in pathogenesis remains to be defined. In vitro expression of *V. cholerae* PilA has, however, not been reported.

*V. vulnificus* PilA is also required for biofilm formation on abiotic surfaces. The role of type IV pili and capsule expression in biofilm development and formation has been described in both *P. aeruginosa* and *V. cholerae* (24, 38, 68). Data from studies in several bacteria have established that type IV pili are important for bacterial adhesion to eukaryotic cell surfaces and pathogenesis and suggest an overlap in factors required for attachment to abiotic surfaces and bacterial adhesion to biotic surfaces (2, 5, 43, 70). Recent studies by Joseph and Wright suggest that in contrast to the role of capsule in other gram-negative bacteria, including *V. cholerae*, the expression of capsule in *V. vulnificus* actually inhibits biofilm formation (18). Studies are currently under way to examine the contribution of pili and capsule in biofilm formation.

In this study, we observed pili on the surface of the *pilA* mutant strain that could not be distinguished from the wild-type strain when examined by electron microscopy. The results of the Western blot analysis show that *pilA* encodes a protein that is processed by PilD. PilA was also expressed as a pilus

structure in a *P. aeruginosa* nonpiliated mutant strain. Examination of the genomes of the two sequenced *V. vulnificus* strains shows the presence of a second type IV pilin, MshA (3, 21), that may also be processed by *pilD* and likely accounts for the pili observed on the surface of the *pilA* mutant strain. In our previous study, pili were not observed on the surface of the *pilD* mutant strain, implying that any pilin precursors in *V. vulnificus* are processed by PilD and are members of the type IV class (40). The absence of a second type IV prepilin peptidase gene in the *V. vulnificus* genome strongly suggests that in addition to PilA and the type II secretion pseudopilins, PilD processes the second type IV pilin, MshA (58). The role of *V. vulnificus* PilD in processing multiple prepilins as well as other prepilin-like proteins from the type II secretion system is analogous to the role of PilD/VcpD, the type IV prepilin peptidase in *V. cholerae*, which is required for assembly of the MSHA and PilA pilins as well as activation of several proteins that are part of the *esp* locus (10, 29).

The genetic organization of *V. vulnificus pilABCD* closely resembles that of the *pil* gene clusters of *V. cholerae* (10, 67) and *P. aeruginosa* (36), the *tap* gene clusters of *A. hydrophila* (41) and *A. salmonicida* (30), and the type IV-A pilus assembly genes of *E. coli* (69). This includes the presence of a *nadC* homologue upstream of *pilA*, as in *V. cholerae* and *E. coli* (10, 67, 69). The genes encoding the pilin, *pilA* in *P. aeruginosa* and *tapA* in *A. salmonicida*, are required for virulence, although a positive correlation with a pilus structure has only been shown in *P. aeruginosa* (4, 11, 37, 48, 57, 59, 61).

The *pilA* and *pilD* genes are highly conserved in clinical as well as environmental isolates of *V. vulnificus*, implying that these genes and consequently type IV pili likely have an essential role in the ecology of this organism. PilA clearly has a role in biofilm formation, adherence to human epithelial cells, and in the virulence of *V. vulnificus.* The bacterium naturally and asymptomatically colonizes shellfish such as oysters (9, 71), and transcription and expression of PilA may conceivably be modulated by environmental conditions. Further studies are necessary to elucidate the role of these pili in oysters and in the environment. The characterization of the other *V. vulnificus* type IV pilus will considerably aid in the clarification of this role.

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