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Genetic Characterization of a New Type IV-A Pilus Gene Cluster Found in Both Classical and El Tor Biotypes of *Vibrio cholerae*

KARLA JEAN FULLNER[1] AND JOHN J. MEKALANOS[1,2]*

Department of Microbiology and Molecular Genetics[1] and Shipley Institute of Medicine,[2] Harvard Medical School, Boston, Massachusetts 02115

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The *Vibrio cholerae* genome contains a 5.4-kb *pil* gene cluster that resembles the *Aeromonas hydrophila tap* gene cluster and other type IV-A pilus assembly operons. The region consists of five complete open reading frames designated *pilABCD* and *yacE*, based on the nomenclature of related genes from *Pseudomonas aeruginosa* and *Escherichia coli* K-12. This cluster is present in both classical and El Tor biotypes, and the *pilA* and *pilD* genes are 100% conserved. The *pilA* gene encodes a putative type IV pilus subunit. However, deletion of *pilA* had no effect on either colonization of infant mice or adherence to HEp-2 cells, demonstrating that *pilA* does not encode the primary subunit of a pilus essential for these processes. The *pilD* gene product is similar to other type IV prepilin peptidases, proteins that process type IV signal sequences. Mutational analysis of the *pilD* gene showed that *pilD* is essential for secretion of cholera toxin and hemagglutinin-protease, mannose-sensitive hemagglutination (MSHA), production of toxin-coregulated pili, and colonization of infant mice. Defects in these functions are likely due to the lack of processing of N termini of four Eps secretion proteins, four proteins of the MSHA cluster, and TcpB, all of which contain type IV-A leader sequences. Some *pilD* mutants also showed reduced adherence to HEp-2 cells, but this defect could not be complemented in *trans*, indicating that the defect may not be directly due to a loss of *pilD*. Taken together, these data demonstrate the effectiveness of the *V. cholerae* genome project for rapid identification and characterization of potential virulence factors.

Vibrio cholerae is a gram-negative bacterial pathogen that causes the waterborne diarrheal disease cholera. Following ingestion by a host and entry into the upper intestine, *V. cholerae* colonizes the intestinal mucosa and begins to export enterotoxins, including the major virulence toxin, cholera toxin (CT). The activity of CT elicits severe diarrhea in the infected host, resulting in extreme dehydration, the hallmark of cholera.

Although extensive research has elucidated the key features of toxin production and regulation, the basic mechanism underlying the initial colonization of the intestine by *V. cholerae* remains elusive. Much work to date has focused on the identification of *V. cholerae* pili. *V. cholerae* produces at least three morphologically distinct types of pili (17). The first type, the toxin-coregulated pili (TCP), are bundle-forming pili that are coordinately regulated with CT (53). These pili are absolutely essential for colonization of the intestine by *V. cholerae*, both in the infant mouse colonization model and in human volunteers (2, 20, 40, 50, 53, 55). However, several observations suggest that TCP may not be directly required for adherence. Purified TCP do not bind to human intestinal epithelium, and the adherence of *V. cholerae* to intestinal epithelium and various epithelial cell lines is not blocked by growth under non-TCP-inducing conditions or by anti-TCP antibodies (3, 12, 48, 51). Further, when classical *V. cholerae* strains are grown under TCP-expressing conditions, the bacteria aggregate, suggesting that TCP plays a key role in bacterium-to-bacterium adhesion (9, 53). These observations indicate that other *V. cholerae* factors may mediate cellular adherence.

A second type of *V. cholerae* pili, mannose-sensitive hemagglutination (MSHA) pili, has also been investigated. These investigations have been done almost exclusively with El Tor strains because classical strains produce few or no pili (26). Within the El Tor strains, MSHA pili are essential for the hemagglutination of erythrocytes, although no other role in adherence or pathogenesis has been ascribed to these pili (24, 25). Disruption of the gene encoding the primary pilin subunit, *mshA*, has no effect on colonization of infant mice (2, 55) or on disease symptoms in human volunteers (50). Further, spontaneous mutants defective in hemagglutinating activity adhere well to intestinal tissue samples (42, 54). Recently, it was shown that MSHA pili mediate the adherence of *V. cholerae* El Tor strains to solid substrates, suggesting that they are important for survival in the environment rather than in the host (58).

Interestingly, the major pilin subunits of both TCP and MSHA pili, TcpA and MshA, respectively, are members of the type IV protein superfamily (25, 46). The type IV proteins all have recognizable N-terminal leader sequences that specify cleavage and N-methylation by specific prepilin leader peptidases (21). In addition to MshA and TcpA, *V. cholerae* contains at least eight other type IV proteins: EpsG, EpsH, EpsI, and EpsJ, which are part of a type II export machinery that secretes CT and other toxins; MshB, MshC, and MshD, which are part of the MSHA pilus assembly machinery; and TcpB, a protein essential for the production of TCP (25, 31, 40, 44). The *tcpJ* gene encodes a prepilin peptidase that processes TcpA into a form that can be assembled into TCP. Surprisingly, the disruption of *tcpJ* abolishes TCP assembly but does not affect either toxin secretion or hemagglutination of erythrocytes (28). This result suggests that a second prepilin peptidase is responsible for processing of the Eps and Msh proteins (28). The identification of a third type of pili by electron microscopy studies also suggests that another, uncharacterized pilus may be present in *V. cholerae* (17).

* Corresponding author. Mailing address: Department of Microbiology and Molecular Genetics, Harvard Medical School, Building D1-427, 200 Longwood Ave., Boston, MA 02115. Phone: 617-432-1935. Fax: 617-738-7664. E-mail: jmekalanos@hms.harvard.edu.

TABLE 1. *V. cholerae* strains used in this study[a]

Strain	Description of genotype and construction	Reference(s) or source
O395	Wild-type classical biotype; Sm^r	Laboratory strain
N16961Sm	Spontaneous Sm^r isolate of wild-type El Tor biotype N16961	The Salk Institute and this study
O395N1	O395 Δ*ctxA* Sm^r	33
P4 (SM44)	P27459 (El Tor) Δ*ctxAB* Sm^r Km^r	14
BGD4	O395 Δ*tcpA* Sm^r	9
KFV5	O395 *pilD*::kanπ; constructed with pKJF306; Sm^r Km^r	This study
KFV5R	KFV5 reverted to faster growth; Sm^r Km^r	This study
KFV5R(pKJF308)	KFV5R complemented with *pilD* in *trans* under the control of the arabinose-inducible promoter P_{BAD}; Sm^r Km^r Ap^r	This study
KFV5R(pKJF308Cm)	KFV5R complemented with *pilD* in *trans* under the control of the arabinose-inducible promoter P_{BAD}; Sm^r Km^r Cm^r	This study
KFV6	N16961Sm Δ*pilA*; constructed with pKJF313; Sm^r	This study
KFV8	O395 Δ*pilA*; constructed with pKJF313; Sm^r	This study
KFV9	O395N1 Δ*pilA*; constructed with pKJF313; Sm^r Km^r	This study
KFV10	N16961Sm Δ*lacZ*; constructed with pDLT; Sm^r	This study
KFV11	N16961Sm Δ*mshA*; constructed with pHT1; Sm^r	58
KFV12	N16961Sm Δ*pilA* Δ*mshA*; constructed from KFV11 with pHT1; Sm^r	This study
KFV16	O395N1 Δ*lacZ*; constructed with pDLT; Sm^r Km^r	This study
KFV18	N16961Sm *pilD*::kanπ; constructed with pKJF306; Sm^r Km^r	This study
KFV18R	KFV18 reverted to faster growth; Sm^r Km^r	This study
KFV18R(pKJF308)	KFV18R complemented with *pilD* in *trans* under the control of the arabinose-inducible promoter P_{BAD}; Sm^r Km^r Ap^r	This study
KFV26	P4 Δ*lacZ*; constructed with pDLT; Sm^r Km^r	This study
KFV32	O395 Δ*pilD*; constructed with pKJF324; Sm^r	This study
KFV33	N16961Sm Δ*tcpA*; constructed with pHT3; Sm^r	This study
KFV36	N16961Sm Δ*pilA* Δ*pilD*; constructed from KFV6 with pKJF324; Sm^r	This study
KFV38	P4 Δ*pilA*; constructed with pKJF329; Sm^r Km^r	This study
KFV43	N1691Sm Δ*hapA*; constructed with pCVDΔHapSal; Sm^r	This study
KFV44	KFV18R Δ*hapA*; constructed from KFV18R with pCVDΔHapSal; Sm^r Km^r	This study
KP8.97	O395 *tcpB*::Tn*phoA*; Sm^r Km^r	40
KP9.79	O395 *tcpA*::Tn*phoA*; Sm^r Km^r	40
Lac1	O395 Δ*lacZ*; reconstruction of CG842 (12); Sm^r	56a
O395(CTXϕ704A)	O395 carrying the replicative form of transducing phage CTXϕ704A; Sm^r Ap^r	30a
Peru-15(CTXϕ-Km)	Peru-15 carrying the replicative form of transducing phage CTXϕ-Km; Sm^r Km^r	29, 57

[a] For a more detailed description of strain construction, see Materials and Methods.

Recent progress by The Institute for Genomic Research (Gaithersburg, Md.) on the *V. cholerae* genome project has facilitated the rapid identification of genes of particular interest. In this paper, we describe the use of early data releases to identify a new gene cluster that is similar to type IV-A pilus assembly gene clusters. Proteins encoded by this gene cluster include a second prepilin peptidase that is important for toxin secretion, MSHA, and TCP production, as well as a new, putative type IV pilin protein. However, a role in pathogenesis for this pilin protein could not be established.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *V. cholerae* strains used in this study are listed in Table 1. *Escherichia coli* DH5α and DH5αλpir were used for

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Location (nt)[a]	Sequence (5′-3′)[b]
NADC1	9–31	CTTGAGATGATGCGTGAAGCGGT
PRO1	435–418	CATAATTTCCTCCGTAG
PILA1	869–891	GTACTGCAGGTGCAACAATTAAC
PILA2	432–453	AGTCATATGAAAGCGTATAAGAACAAA
PILA3	891–871	GGTGAGCTCGTTAATTGTTGCACCTGCAGT
PILB1	894–914	ACACCTACGGAGGAAATTATGCTCACCAAC CTTGTTGCT
PILB2	1520–1543	CATAGCCTAGGCGGACGCGACGC
PILB3	1397–1421	CTGCAATCCATTGAAGATCTCAGC
PILB4	1978–2000	AATACCAAGTGCAAGTGCAGCCG
PILB5	1695–1672	AATGGCGGTATCGCCCGTTAGGC
PILC1	3442–3420	GAAGCCTTAGCGAGCACATTACC
PILC2	2905–2881	CCAACTTGAGGGCTTGCACAATGG
PILC3[c]	2385–2361	AATAGCCGCCCGTACACTCGTTAC
PILC4	2664–2687	TGGAAGGCATCAACAGCAACGG
PILC5	3192–3213	ATGCTCACCATGGTCATCCCAG
PILC6	3911–3889	CATTCTCTTGCTCTAAGCGAAT
PILD1	3891–3911	GAAATTCGCTTAGAGCAAGAGAATG
PILD2	4796–4773	ACGCGTCGACAAAACTCATAACGGTTG
PILD3	4318–4327	GCTATGTGTTGATTGCGGCA
PILD5	4045–4022	CGTGATTCCGCGCGCCATTCACG
YACE1	4780–4802	ATTCGCTTAGAGCAAGAGAATGAGTTTTGT CGTAGCATTGA
YACE2	5394–5371	ATTCTCACAAATTTTGCCTGCTC

[a] According to the *pil* operon sequence determined for this paper.
[b] Underlined nucleotides are not exact matches to the sequence and were altered either to add restriction enzyme sites or to facilitate crossover PCR.
[c] Actually binds within the *pilB* open reading frame.

construction of plasmids. SM10λpir and β2115 were used to deliver plasmids to *V. cholerae* by conjugation. All strains were grown on Luria-Bertani (LB) medium at 37°C, except as noted otherwise. Antibiotics (micrograms per milliliter) used were as follows: ampicillin, 50; chloramphenicol, 4; kanamycin, 50; and streptomycin, 100.

PCR and DNA sequencing. All oligonucleotides used for PCR and DNA sequencing were obtained from Biosynthesis, Inc. (Lewisville, Tex.) or Operon Technologies (Alameda, Calif.) and are listed in Table 2. PCR was performed with TaKaRa *Taq* polymerase and reagents from Oncor (Gaithersburg, Md.), PCR Supermix from Gibco BRL (Gaithersburg, Md.), or Vent *Taq* polymerase and reagents from New England BioLabs (Beverly, Mass.). PCR templates were prepared by suspending a single bacterial colony in 200 μl of distilled H_2O, of which 1 μl was used in either a 25- or a 50-μl PCR amplification. For sequencing of the *pil* operon and for preparation of all plasmids, colonies of *V. cholerae* N16961 or N16961Sm were used as the PCR template, except as noted otherwise. All PCRs were performed with standard amplification parameters and an MJ Research Thermocycler (model PTC-200). For direct sequencing, PCR products were purified by two passages over a QiaQuick PCR purification kit (Qiagen, Valencia, Calif.). Automated DNA sequencing was done at the Harvard Microbiology Core Sequencing Facility. The reported sequence has a minimum of fourfold coverage with at least two sequences for both the coding and the noncoding strands. The DNA sequence was assembled and open reading frames were assigned with the GeneWorks Analysis software package.

Construction of counterselectable plasmids. pKJF306 is a streptomycin-based counterselectable plasmid which generates kanamycin insertions in *pilD*. Primers PILD1 and PILD2 were used in a *Taq* polymerase PCR to generate a 908-bp product encompassing the *pilD* gene; this fragment was ligated directly into the TA cloning vector pCR2.1 (Invitrogen, Carlsbad, Calif.) to create pKJF302. The fragment was then moved to pBluescript IIKS(+) (Stratagene, La Jolla, Calif.) as an *Eco*RI fragment to generate pKJF303. The kanamycin resistance cassette kanπ (Pharmacia, Piscataway, N.J.) as an *Hae*III fragment and inserted into *pilD* on pKJF303 between two *Eco*47III sites found at nucleotides (nt) 294 and 334 of the *pilD* PCR product to generate pKJF305. The entire *pilD*::kanπ fragment was then moved into the streptomycin-based counterselectable plasmid pKAS32 (47) as an *Eco*RI fragment, generating plasmid pKJF306.

Counterselectable plasmids pKJF313 and pKJF329 were used to generate nonpolar deletions of *pilA*. The first step in the construction of these plasmids was crossover PCR. PCR with primers NADC1 and PRO1 amplifies the region 428 bp upstream of the putative translational start site of *pilA*, while PILB1 and PILB2 amplify the region 647 bp downstream of *pilA*. Primers PRO1 and PILB1 have 21 bp of overlapping nucleotide sequence, such that the crossover PCR brings the second codon of *pilB* to the AUG codon of *pilA*. PCR products resulting from Vent *Taq* polymerase PCR with primer pair NADC1-PRO1 or PILB1-PILB2 were purified by two passages over the QiaQuick PCR purification kit. Five microliters of each product was then added to 45 μl of PCR Supermix and treated in a 10-cycle reaction (92°C, 40 s; 50°C, 40 s; 72°C, 45 s) without primers to enhance crossover extension of the two products. One microliter of this reaction mixture was then used as a template with PCR Supermix and NADC1 and PILB2 as primers to amplify the 1,075-bp crossover product, which was then directly ligated into pCR2.1 to create pKJF310. The crossover fragment was then moved to the polylinkers of the streptomycin-based counterselectable plasmid pKAS46 (47) and the sucrose-based counterselectable plasmid pWM91 (34) as a *Spe*I-*Xho*I fragment to generate pKJF313 and pKJF329, respectively.

Counterselectable plasmid pKJF324 generates a nonpolar deletion of *pilD*. This plasmid was made following the crossover PCR protocol described above. In the first reaction, primers PILC5 and PILC6 amplify the region 850 bp upstream of *pilD*, while YACE1 and YACE2 amplify the region 750 bp downstream of *pilD*. YACE1 and PILC6 share 21 nt of homology such that the second codon of *yacE* is linked to the first AUG codon of *pilD* in the crossover PCR. Following crossover PCR, the 1,800-bp product was cloned into pCR2.1 and was then moved to the polylinker of sucrose-based counterselectable plasmid pWM91 (34) as a *Spe*I-*Xho*I fragment to create pKJF324.

Construction of *pilD*-complementing plasmids. Primers PILD1 and PILD2 are designed to introduce *Eco*RI and *Sal*I sites at the 5′ and 3′ ends of *pilD*, respectively. Following PCR amplification, the 908-bp product was treated for 20 min at 72°C with *Taq* polymerase to add an adenosine nucleotide to the termini for direct cloning into pCR2.1 to create pKJF304. The novel *Eco*RI and *Sal*I sites were then used to move *pilD* into the pBAD18 (15) polylinker, placing *pilD* under the control of the arabinose-inducible promoter P_{BAD} but with its own ribosome binding site. For alternative antibiotic selection, the *pilD* insert was moved from pBAD18 to pBAD18Cm (15) by use of a unique *Mlu*I site found on both plasmids and *Sal*I from the polylinker. The resulting plasmids, pKJF308 and pKJF308Cm, were transferred to *V. cholerae* by electroporation.

Generation of mutations in *V. cholerae* strains by use of counterselectable markers. Counterselectable plasmids pKJF306 (pKAS32 *pilD*::kanπ), pKJF313 (pKAS46 Δ*pilA*), pKJF324 (pWM91 Δ*pilD*), pKJF329 (pWM91 Δ*pilA*), pDLT (pCVD442 Δ*lacZ* [57a]), pHT1 (pCVD442 Δ*mshA1* [55]), pHT3 (pCVD442 Δ*tcpA10* [55]), and pCVDΔHapSal (pCVD442 Δ*hapA* [17a]) were introduced into *V. cholerae* by conjugation on an LB agar plate. Counterselections were done by the protocol of Metcalf et al. (34). Briefly, four cointegrants were purified by streaking one time under selection and then were passaged one time without selection to allow recombination to occur. Sixteen independent colonies were then streaked on the counterselection medium: LB medium containing streptomycin at 3 mg/ml for streptomycin-based selection and LB medium without NaCl but containing 6% sucrose for sucrose-based selection. Best results were achieved when counterselection plates were incubated at room temperature for 2 days. Isolated colonies were checked by PCR for gain of the mutation and loss of the wild-type gene.

Agg and CTXφ transduction. Classical *V. cholerae* cultures were grown in 5 ml of LB broth at 30°C with rolling for optimal production of TCP (11). The autoagglutination (Agg) phenotype was scored visually. For CTXφ transductions, phage were prepared by filtering culture supernatant of Peru-15(CTXφ-Km) or O395(CTXφ704A) with a 0.2-μm-pore-size syringe filter. Fifty microliters of phage preparation was mixed with 50 μl of TCP-expressing culture, and the mixture was allowed to stand for 45 min at room temperature to allow for infection by the phage and expression of antibiotic resistance genes. The mixture was then diluted and plated. The transduction frequency is expressed as the CFU of transductants over the CFU of the recipient strain.

CT assay. For optimal toxin production, classical strains and El Tor strains containing plasmid pBAD18Cm-ToxT (19) were grown at 30°C with rolling for 16 h. One milliliter of culture was spun down in a microcentrifuge, and the culture supernatant was set aside. The bacterial cells were resuspended in 1 ml of 10 mM Tris-HCl–1 mM EDTA–20% glucose (pH 7.5) and sonicated (Branson Sonifier 350) twice for 15 s each time on 20% power. CT present in 100 μl of culture supernatant or sonicate was measured by the G_{M1}-ganglioside enzyme-linked immunosorbent assay with purified CT to generate a standard curve (11).

HAP assays. The presence of hemagglutinin-protease (HAP) was detected on LB agar plates containing 1% nonfat milk (37). For some assays, 4 ml of agar was solidified in divided glass plates such that different quadrants contained different antibiotics and/or inducing compounds.

MSHA assays. MSHA assays were performed as previously described, except that washed defibrinated sheep blood was substituted for human or chicken erythrocytes (12).

Infant mouse colonization. Overnight cultures of *V. cholerae* strains were diluted 1:100 in fresh LB medium and grown with rolling to the mid-log to late log phase. Cultures were diluted in 0.15 M NaCl, and 8 μl of blue food coloring was added. Five- to 7-day-old infant mice were taken from their mothers at least 5 h prior to oral infection. Fifty microliters of inoculum was delivered orally to anesthetized mice. Mice were kept at 30°C for a full 24 h, at which time they were sacrificed and their intestines were dissected from above the cecum. The intestines were homogenized in 5 ml of LB broth, diluted, and plated on LB agar with streptomycin and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). For competition assays, CFU of blue (mutant) and white (wild-type) strains were counted, and the colonization index was calculated as the ratio of blue colonies to white colonies.

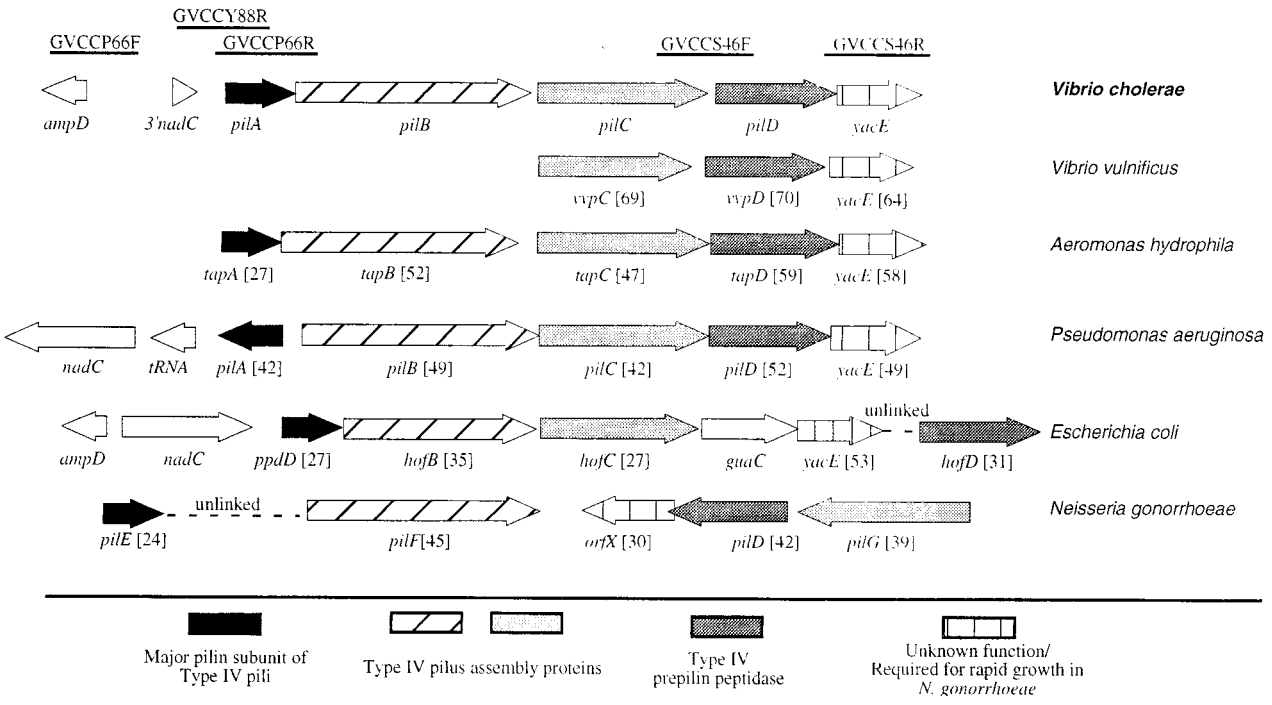

FIG. 1. Organization of six representative type IV pilus assembly operons. The bars at the top indicate the approximate locations and designations of DNA sequences obtained from the publicly available genomic database. Numbers in brackets indicate the percentages of amino acid identity of deduced protein sequences to the translated open reading frame of the appropriate *V. cholerae* gene. Percentages of identity were determined by a ClustalW alignment done with a MacVector software package. Sequences were assembled from GenBank and other public databases as follows: *V. vulnificus* (accession no. AF070934) (38), *A. hydrophila* (accession no. U20255) (39), *P. aeruginosa* (accession no. M32066 and M14849) (23, 35) (genomic data from www.pseudomonas.com), and *E. coli* (accession no. L28105, AE000409, and D26562) (4, 59), and *N. gonorrhoeae* (accession no. U32588 and X66144) (10, 43, 56).

HEp-2 cell adherence assays. Overnight cultures of *V. cholerae* strains were diluted 1:100 in fresh LB medium and grown at 37°C with rolling to the mid- to late-log phase. Bacterial cells were washed in phosphate-buffered saline (PBS) and adjusted to 10^9 bacteria per ml. Ten microliters was added to HEp-2 epithelial cells (ATCC F-13959) that had been grown in RPMI 1640 medium containing 10% fetal calf serum without antibiotics in 24-well dishes to 10^5 cells per well. For classical strains, plates were spun for 10 min at 300 × *g* and incubated at 37°C in 5% CO_2 for 1 h. Cells were washed four times with PBS, and adherent bacteria were recovered in 1 ml of PBS–0.1% Triton X-100. For El Tor strains, plates were spun for 2 min and incubated for 15 min to reduce nonspecific adherence to the plastic. Cells were washed and recovered in 1 ml of PBS–1.0% Triton X-100. Percent adherence was calculated as CFU adherent divided by CFU added times 100.

Nucleotide sequence accession number. The nucleotide sequence reported in this study was assigned GenBank accession no. AF109904.

RESULTS

Bioinformatics and sequencing of the *pil* gene cluster of *V. cholerae*. The publicly available sequences from the *V. cholerae* genome project (56a) were examined for the presence of open reading frames with known functions in pathogenesis. Interestingly, sequence runs GVCCS46F and GVCCS46R contain sequences with similarity to two genes found in type IV-A pilus assembly operons (Fig. 1). In addition, sequence runs GVCCP66R and GVCCY88R encode a novel type IV cleavable pilin protein that is closely related to the type IV-A pilin subunit of *Pseudomonas aeruginosa* (Fig. 1). In order to show that sequences GVCCP66R and GVCCS46F are linked on the *V. cholerae* genome, primers PILA1 and PILC1 were used in a PCR to amplify a 2.6-kb product representing the intervening region. Primers PILD1 and PILD2 were used to generate a 908-bp product to demonstrate the linkage of GVCCS46F and GVCCS46R (data not shown). The entire 5,401-bp gene cluster was sequenced directly from progressively smaller PCR products such that the operon was sequenced with a minimum of fourfold coverage with at least two sequences for each strand. All sequences from the original data release by The Institute for Genomic Research were also confirmed by the same method.

The *V. cholerae pil* gene cluster resembles other type IV-A pilus gene clusters. The 5,401-bp region of *V. cholerae* contains five complete open reading frames, apparently organized into a single operon. The designation *vcpD* has been proposed for the fourth gene of this cluster (32), based on the nomenclature of the *V. vulnificus* operon (38). However, the name *vvpA*, appropriate for the first gene by this nomenclature, has already been used to describe a *V. vulnificus* protease gene (6). To avoid further confusion, the nomenclature for *P. aeruginosa*, the organism with the first identified type IV pilus operon, has been adopted for the first four open reading frames, *pilABCD*, and the fifth gene is referred to as *yacE*, consistent with the *E. coli* genome project designation. As a whole, the cluster is referred to as the *pil* gene cluster.

The organization of the *V. cholerae pil* gene cluster shows a striking similarity to that of the *Aeromonas hydrophila tap* gene cluster (Fig. 1). Although other organisms also tend to group these five genes, *V. cholerae* and *A. hydrophila* are apparently the only organisms which group these genes into a single operon. Presumably, the *V. vulnificus* operon is similarly arranged, although only *vvpC*, *vvpD*, and *yacE* have been identified to date. Interestingly, like the type IV-A pilus assembly operon from *E. coli*, the *V. cholerae pil* gene cluster is linked to *ampD* and *nadC*, demonstrating that the arrangement of these genes is well conserved between these two gram-negative species.

The first open reading frame, *pilA*, has two potential translation start sites. The second start site was selected because a

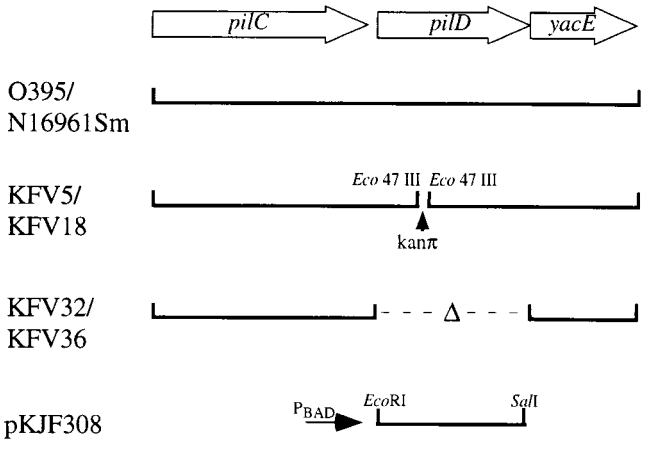

FIG. 2. Genetic organization of *pilD* mutants and the complementing plasmid. Construction of strains and plasmids is detailed in Materials and Methods. In addition to the *pilD* deletion, KFV36 also has a deletion in *pilA*.

consensus ribosome binding site is located just upstream of the AUG, whereas no such site is present upstream of the first potential start site. Further, consistent with the grouping of *V. cholerae* PilA into the type IV-A family, the use of the second translation start site would give a type IV signal sequence of 11 amino acids. Use of the first potential translation start site would give PilA a long signal sequence of 25 amino acids, a feature more common in type IV-B pilin subunits, such as *V. cholerae* TcpA (13).

Although the amino acid sequence similarity among the type IV-A pilins is generally localized to the first 30 amino acids, the sequence of the *pilA* gene from classical strain O395 is 100% identical at both the protein and the nucleotide levels to the El Tor strain N16961 gene sequence, indicating that the *pilA* gene is highly conserved in *V. cholerae*.

The next two open reading frames, *pilB* and *pilC*, are likely to encode proteins essential for the assembly of a new type IV pilus (1). Recent work with *P. aeruginosa* has shown that *pilB* and *pilC* are merely 2 of up to 30 genes essential for the assembly of type IV pili. These genes include *pilT*, *pilE*, and *pilU* (1), for which open reading frames are also present in the *V. cholerae* genome (56a).

The fourth open reading frame encodes a putative member of the type IV prepilin peptidase family. These proteins recognize the type IV signal sequence and cleave the signal at a conserved glycine (36). The peptidase then modifies the next residue to the form that is assembled into either pili or other membrane structures (49). Like the *pilA* gene, the *V. cholerae pilD* gene is 100% conserved between the classical strain O395 and the El Tor strain N16961Sm.

The fifth open reading frame, *yacE*, is a member of a family of genes whose localization with type IV pilus assembly gene clusters is frequently conserved (Fig. 1), although its function is unknown.

Effect of various *pilD* mutations on growth patterns. To test whether *pilD* encodes a prepilin peptidase essential for secretion and hemagglutination, we constructed a *pilD* mutant in which the kanamycin resistance cassette, kanπ, was inserted into the *pilD* gene by double homologous recombination with the streptomycin-based counterselectable plasmid pKJF306. Following mating into O395 and N16961Sm, cointegrants were counterselected on streptomycin and kanamycin to select for loss of the integrated plasmid and for maintenance of the kanπ cassette. However, following the selection process, only pinpoint colonies were recovered for both O395 and N16961Sm parental strains. These slowly growing mutants, designated KFV5 and KFV18 (Fig. 2), respectively, reverted to a faster growth pattern with a high frequency when grown in liquid cultures. Revertants for these strains were single colony purified and designated KFV5R and KFV18R, respectively.

It was first surmised that the growth defect may be caused by the loss of the downstream gene *yacE*. Like the *V. cholerae pilD* insertion, transposon insertions in *Neisseria gonorrhoeae pilD* result in slow growth, but this slow growth is associated with the loss of the downstream gene *orfX*, a gene similar to *V. cholerae yacE* (10). A nonpolar *pilD* mutant of O395 was made by deleting the kanamycin cassette from KFV5 by use of the

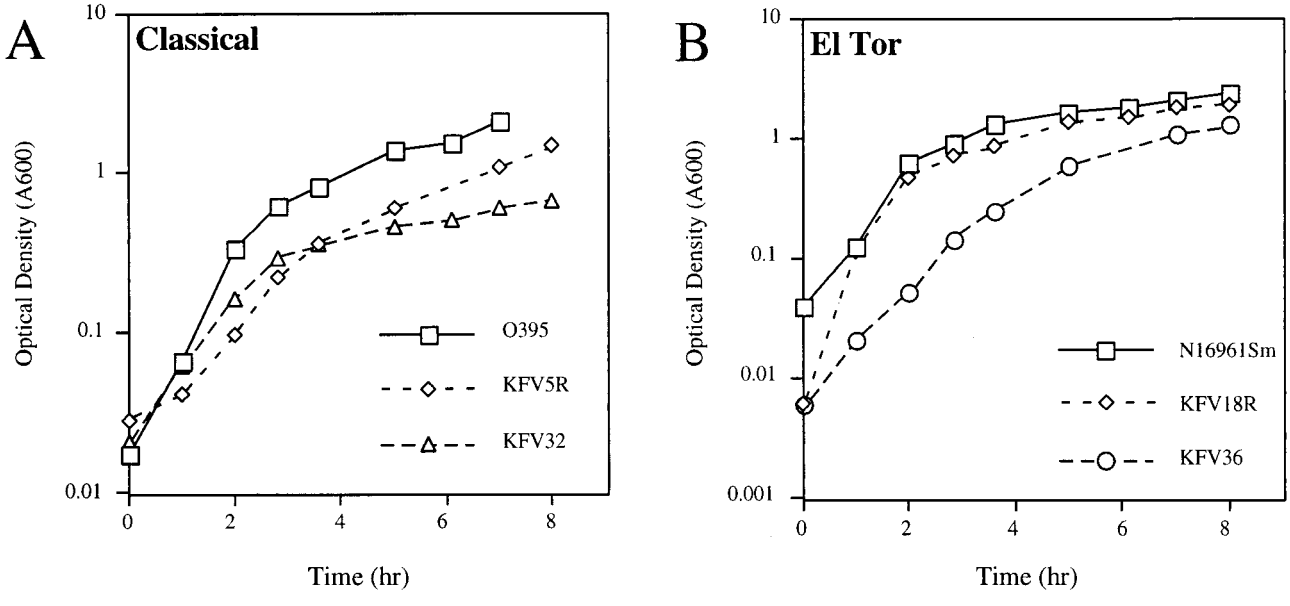

FIG. 3. Growth curves for *pilD* mutants. Overnight cultures were diluted 1:100 in 100 ml of LB broth with antibiotics and incubated with shaking at 37°C. At specific times, 1 ml of the cultures was removed and the optical density (A_{600}) was measured. Time zero is the time at which the cultures exited the stationary phase.

sucrose-based counterselectable plasmid pKJF324. As shown in Fig. 3A, the resulting strain, KFV32, grew slowly in cultures, demonstrating an apparent premature entry into the stationary phase. The revertant, the kanamycin insertion mutant KFV5R, although continuing to grow more slowly than the wild type, apparently escaped this premature stationary phase (Fig. 3A). In contrast, revertant KFV18R, an El Tor mutant, grew with a pattern similar to that of the wild type, suggesting that the mechanism of reversion in these two strains may differ.

Unfortunately, attempts to create a nonpolar deletion of *pilD* in El Tor strain N16961Sm by either deletion of *pilD* from the wild-type background or deletion of the *kan*π insertion were not successful. However, it has been reported that slowly growing *pilD* mutants of *N. gonorrhoeae* can revert to faster growth by accumulating deletions in the pilin subunit gene, *pilE* (10). In accordance with this result, a nonpolar deletion of the *pilD* gene could be constructed in a *pilA* deletion background. However, as shown in Fig. 3B, this mutant, designated KFV36, grew more slowly than the wild type. Also, deletion of *pilA* from the classical deletion mutant KFV32 did not enhance its growth beyond that shown in Fig. 3A (data not shown). Thus, it seems that, in the absence of *pilD*, PilA precursor proteins are not processed and contribute in part, but not entirely, to slow growth.

***V. cholerae pilD* is essential for the secretion of CT and HAP.** The *eps* operon of *V. cholerae* encodes a type II secretion system that is essential for the export of CT, HAP, and other *V. cholerae* toxins (37, 44). Four of the proteins encoded by this operon, EpsG, EpsH, EpsI, and EpsJ, contain type IV signal sequences, although a gene for a type IV peptidase is absent from this operon (44). The signal sequence of EpsG is cleaved by *N. gonorrhoeae* PilD but not by *V. cholerae* TcpJ (44). These observations suggest that the newly recognized *pilD* gene of *V. cholerae* may encode a prepilin peptidase essential for toxin secretion.

To determine if *pilD* of *V. cholerae* is essential for the processing of EpsG, EpsH, EpsI, and EpsJ, the various *pilD* mutants were analyzed for defects in the secretion of CT and HAP. As shown in Fig. 4A, wild-type classical strain O395 exported 97% of the CT that it produced. In contrast, most of the CT produced by mutants KFV5R and KFV32 accumulated within the bacteria and was not exported (Fig. 4A). Export could be restored by the introduction of *pilD* on a plasmid under the control of the arabinose-inducible promoter P_{BAD}. The export of CT was not complemented either by the pBAD18 vector alone or in the absence of the inducer arabinose (data not shown).

El Tor *pilD* mutants also had defects in CT export. To increase the expression of CT, N16961Sm and KFV18R were transformed with plasmid pBAD18Cm-ToxT. The resulting strains overexpressed the regulatory protein ToxT and thereby had increased CT production in standard growth media. Even under these improved induction conditions, KFV18R produced about 10-fold less toxin than the wild type (data not shown). However, the majority of the toxin produced was not secreted, demonstrating that KFV18R had a generalized defect in CT secretion similar to that in the classical *pilD* mutants KFV5R and KFV32 (Fig. 4).

In addition to the loss of CT export, El Tor *pilD* mutants were also defective for the export of a second toxin, HAP. As shown in Fig. 5, HAP could be detected as a zone of clearing around colonies grown on agar plates containing 1% nonfat milk. The zone of clearing was decreased when the structural gene for HAP, *hapA*, was disrupted (Fig. 5, left). Similarly, *pilD* mutant KFV18R showed a decreased zone of clearing. Export could be restored by the introduction of *pilD* in *trans* only in the presence of the inducer arabinose and was not restored by the presence of the pBAD18 vector (Fig. 5, right). Similar results were found for the Δ*pilA* Δ*pilD* double mutant KFV36 (data not shown), although the small-colony phenotype of this strain alone could account for this defect, since HAP expression is proposed to be regulated by quorum sensing (22).

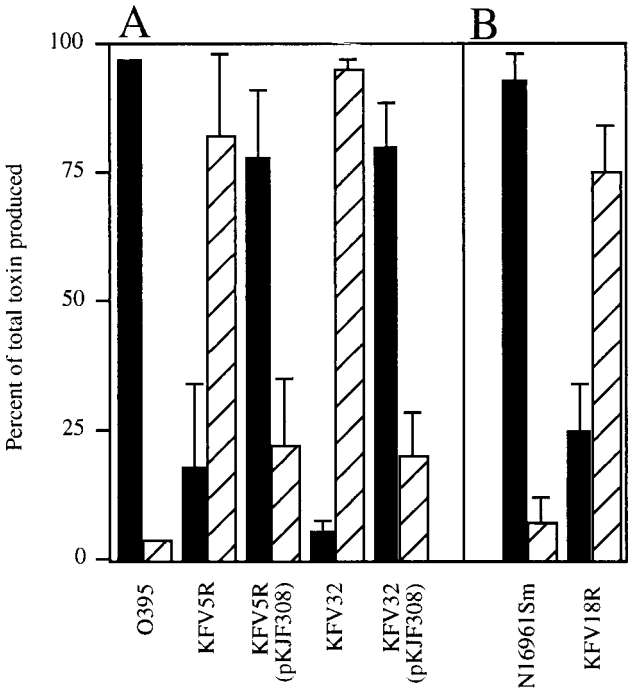

FIG. 4. Effect of *pilD* mutations on CT secretion. CT was measured as described in Materials and Methods. Strains carrying a *pilD*-complementing plasmid were grown in 0.05% arabinose to induce the expression of promoter P_{BAD}, as were N16961Sm and KFV18R, which contain plasmid pBAD18Cm-ToxT for constitutive overexpression of CT. For both classical (A) and El Tor (B) strains, enzyme-linked immunosorbent assay plates were read with a microtiter reader, and amounts (nanograms) of intracellular (hatched bars) and extracellular (solid bars) toxins were determined by comparison against a standard curve. Results are presented as a percentage of the total toxin produced. Averages and standard deviations were determined from duplicates in a single experiment that is representative of at least two experiments for each sample.

Taken together, these data show that *pilD* mutants of both classical and El Tor strains have a generalized defect in toxin export that is due solely to *pilD*, since the mutant phenotype could be complemented in *trans*. This defect is probably directly due to the failure of a *pilD*-encoded peptidase to process EpsG, EpsH, EpsI, and EpsJ.

El Tor *pilD* mutants are defective in MSHA. El Tor strains of *V. cholerae* produce a pilus that can hemagglutinate erythrocytes, but not in the presence of mannose (24). The major subunit of this pilus, MshA, and three accessory pilus assembly proteins, MshB, MshC, and MshD, have type IV cleavable sequences although, similar to the situation with the *eps* operon, a type IV prepilin peptidase is not encoded by the *msh* gene cluster (18, 25, 32). If *pilD* is essential for the construction of the MSHA pilus, then *pilD* mutants should be unable to hemagglutinate erythrocytes.

N16961Sm hemagglutinated sheep erythrocytes, and this hemagglutination was inhibited by both mannose and the nonhydrolyzable analog methyl-α-D-mannopyranoside but not by fucose or arabinose (Table 3). A deletion within the *mshA* gene in N16961Sm abolished this hemagglutination. Similarly, the El Tor *pilD* mutants KFV18R and KFV36 did not hemag-

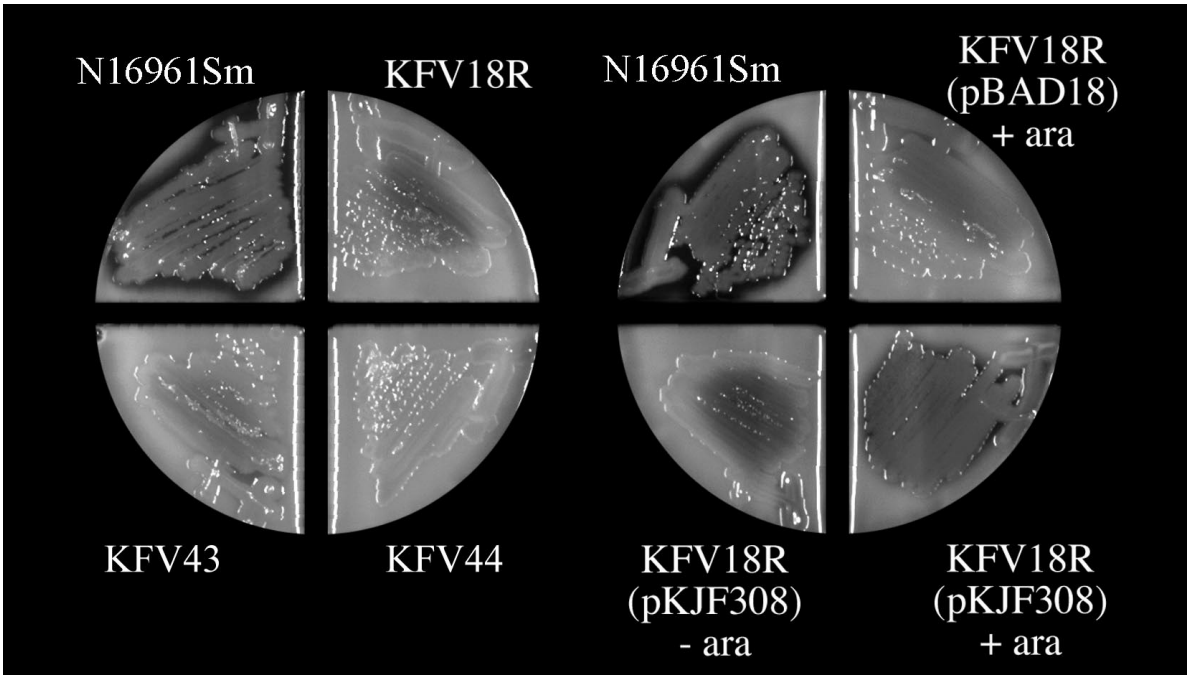

FIG. 5. Effect of *pilD* mutations on HAP secretion. Strains were heavily streaked onto 1% nonfat milk agar in divided glass plates and incubated at 37°C for 24 h. The agar contained 0.05% arabinose (ara) as indicated. Plates were photographed with a Bio-Rad Fluor-S MultiImager.

glutinate erythrocytes. Hemagglutination was restored when these strains were complemented with pKJF308, which carries *pilD* in *trans*, but not when they carried the pBAD18 vector or were grown without arabinose to induce the expression of *pilD* on the plasmid (Table 3).

These data show that *pilD* is essential for MSHA and that the defect is due solely to the loss of *pilD*, since the mutant phenotype could be rescued by the introduction of *pilD* in *trans*. This defect is presumably due to the loss of processing of the pilin subunit MshA as well as the three accessory proteins.

***pilD* mutants do not produce wild-type levels of TCP.** TCP are type IV pili of the B subfamily, members of which typically have longer type IV signal sequences than type IV-A pili and are carried on mobile elements, such as plasmids or pathogenicity islands (13). Since TcpJ processes the major TCP pilin subunit TcpA (28), it is not expected that *pilD* mutants should be defective for TCP production. However, it is possible that the essential accessory protein, TcpB (40), is processed by PilD. If this is the case, *pilD* mutants should be defective for TCP production.

TABLE 3. Effect of *pilD* mutations on MSHA activity

Strain and growth condition	Hemagglutination activity[a]
N16961Sm	+
KFV11 (Δ*mshA*)	−
KFV18R (*pilD*::kanπ, reverted)	−
KFV18R (pBAD18) + 0.05% arabinose	−
KFV18R (pKJF308) + 0.05% arabinose	+
KFV18R (pKJF308) + no arabinose	−
KFV36 (Δ*pilA* Δ*pilD*)	−
KFV36 (pKJF308) + 0.05% arabinose	+
KFV36 (pKJF308) + no arabinose	−

[a] −, no hemagglutination; +, hemagglutination of sheep erythrocytes that was inhibited by mannose but not by fucose or arabinose.

When classical strain cultures are grown under conditions which favor TCP production (11), wild-type strain O395 aggregates, and this aggregation is dependent upon the presence of TCP, since mutants with mutations in *tcpA*, *tcpB*, and other *tcp* genes failed to aggregate (40) (Table 4). Similarly, *pilD* mutants KFV5R and KFV32 failed to aggregate in cultures, demonstrating a general defect in TCP assembly due to mutation of *pilD*.

TCP production can be quantitatively measured by transduction of CTXϕ-Km (kanamycin resistance) and CTXϕ704A (ampicillin resistance), filamentous transducing phages which adsorb to TCP during the infection process (57). Wild-type strain O395 was transduced by both phages at a frequency of 10^{-2}, but *tcpA* and *tcpB* mutants could not be transduced (Table 4). *pilD* mutants KFV5R and KFV32 showed an intermediate phenotype. Although phage transduction was not completely abolished, *pilD* mutants KFV5R and KFV32 were transduced at a 150- to 240-fold-lower frequency (Table 4). Surprisingly, CTXϕ transduction not only was restored but also was slightly enhanced by the presence of *pilD* in *trans*.

These data indicate that *pilD* is very important, although not absolutely essential, for TCP production. We thus propose that TcpA is processed primarily by TcpJ (28), while TcpB is processed primarily by PilD, although residual processing by TcpJ may occur. Further, the overproduction of TCP when the copy number of *pilD* is increased suggests that the processing of TcpB by PilD may be a limiting factor in TCP production.

***pilD* mutants do not colonize infant mice.** Since TCP production is absolutely essential for colonization, *pilD* mutants should not colonize infant mice. Indeed, strains KFV5R and KFV32 did not colonize infant mice when inoculated either by themselves or in competition with a *lacZ* derivative of O395 (Table 5).

Like classical *pilD* mutants, El Tor *pilD* mutant KFV18R did

TABLE 4. Effect of *pilD* mutations on TCP production

Recipient strain and growth condition	Agg	Transduction frequency with[a]:	
		CTXϕ704A	CTXϕ-Km
O395 (TCP-inducing conditions)	+	$9.1 \times 10^{-3} \pm 2.0 \times 10^{-3}$	$9.1 \times 10^{-3} \pm 2.7 \times 10^{-3}$
O395 (non-TCP-inducing conditions)	−		$<2.8 \times 10^{-7} \pm 1.1 \times 10^{-7}$
BGD4 (Δ*tcpA*)	−	$<6.0 \times 10^{-8} \pm 0.1 \times 10^{-8}$	$<4.2 \times 10^{-8} \pm 0.1 \times 10^{-8}$
KP8.97 (*tcpB*::Tn*phoA*)	−	$<9.7 \times 10^{-8} \pm 0.1 \times 10^{-8}$	
KFV5R (*pilD*::kanπ, reverted)	−	$4.1 \times 10^{-5} \pm 0.3 \times 10^{-5}$	
KFV5R (pKJF308Cm) + 0.05% arabinose	+	$4.5 \times 10^{-2} \pm 1.7 \times 10^{-2}$	
KFV5R (pKJF308Cm) + no arabinose	−	$1.0 \times 10^{-3} \pm 0.4 \times 10^{-3}$	
KFV32 (Δ*pilD*)	−		$3.9 \times 10^{-5} \pm 0.4 \times 10^{-5}$
KFV32 (pKJF308) + 0.05% arabinose	+		$1.4 \times 10^{-2} \pm 0.2 \times 10^{-2}$
KFV32 (pKJF308) + no arabinose	−		$2.5 \times 10^{-4} \pm 0.8 \times 10^{-4}$

[a] Transduction frequency is the CFU of transductants divided by the CFU of recovered recipients. Numbers given are averages ± standard deviations from duplicates in a single experiment.

not colonize infant mice reproducibly (Table 5). However, in some experiments, when the highest inocula were used or when the mutant was given at an excess in competition, some colonies of KFV18R could be recovered (Table 5). In similar assays, *tcpA* mutants also were frequently recovered (data not shown). Independent colonies of KFV18R recovered from five mice in two separate experiments were isolated and further analyzed. These isolates maintained the kanπ insertion in *pilD* and continued to be defective for HAP export and MSHA, indicating that they did not arise from in vivo selection for reversion or suppression of the original *pilD* mutations. Further, these mouse-recovered KFV18R isolates did not have enhanced infection capability when inoculated into new infant mice (data not shown). These observations demonstrate that while KFV18R fails to colonize well, the mutant is able to survive the in vivo environment. These observations support the notion that the loss of colonization is due either to the loss of TCP alone or to the loss of another *pilD*-processed protein as well.

***pilA* and *mshA* are not required for the colonization of infant mice.** If the loss of an additional *pilD*-processed protein contributes to the colonization defect, it is possible that the defect is due to *pilA*, which is linked to *pilD*. However, mutants with deletions of *pilA*, in both the classical and the El Tor backgrounds, colonized mice as well as the wild type (Table 6). Similarly, mutants with mutations in the MSHA pilus colonized infant mice as well as the wild type (2, 55) (Table 6). We considered that PilA and MshA might have redundant functions in colonization. However, double Δ*pilA* Δ*mshA* mutants also colonized mice as well as the wild type (Table 6). Since CT itself may play an important role in colonization (41), its presence might mask other adherence factors. However, *pilA* mutants of toxin-deficient strains O395N1 and P4 also showed no defect in colonization compared to the wild type (Table 6). Thus, we conclude that while *pilA* may encode a pilin protein for a new type IV pilus, this putative pilus is not required for colonization in the infant mouse model.

Classical *pilD* mutants adhere poorly to HEp-2 cells due to mutation of an unknown gene. Given that PilD proteins in other organisms process proteins required for epithelial cell adherence (7, 16, 27, 38, 43), we examined the ability of *V. cholerae* mutants to adhere to HEp-2 cells to determine whether potential adherence factors other than TCP are absent in *pilD* mutants. When grown under conditions optimal for adherence, O395 adheres to HEp-2 cells. In contrast, the *pilD* mutant KFV5R adhered poorly compared to the wild type, showing up to a 10-fold defect in repeated assays (Fig. 6A). Surprisingly, unlike other *pilD*-dependent processes, this defect could not be

TABLE 5. Infant mouse colonization by *pilD* mutants of classical and El Tor *V. cholerae* strains

Competing strains[a]	Inoculum ratio[b]	In vitro ratio[c]	In vivo colonization[d]			
			No. of mice	Avg CFU recovered		Ratio (mutant/Δ*lacZ* strain)
				Mutant	Δ*lacZ* strain	
KFV5R and Lac1	0.98	7.9×10^{-5}	4	0	$3.4 \times 10^{6} \pm 4.2 \times 10^{6}$	$<2.9 \times 10^{-7}$
	3.8	1.3×10^{-3}	5	0	$2.3 \times 10^{6} \pm 3.8 \times 10^{6}$	$<4.3 \times 10^{-7}$
	41.3	0.056	4	0	$5.4 \times 10^{5} \pm 4.9 \times 10^{5}$	$<1.9 \times 10^{-6}$
O395N1 alone			5	$1.6 \times 10^{7} \pm 1.8 \times 10^{7}$		
KFV5R alone			5	0		
KFV18R and KFV10	0.39	1.5×10^{-4}	4	0	$1.4 \times 10^{8} \pm 0.9 \times 10^{8}$	$<7.3 \times 10^{-9}$
	2.5	4.0×10^{-4}	4	0	$1.2 \times 10^{8} \pm 1.1 \times 10^{8}$	$<8.6 \times 10^{-9}$
	20.9	0.028	5	$1.6 \times 10^{4} \pm 1.8 \times 10^{4}$	$2.6 \times 10^{7} \pm 2.6 \times 10^{7}$	6.2×10^{-4}
P4 alone			4	$2.5 \times 10^{4} \pm 1.2 \times 10^{4}$		
KFV18R alone			9	$\leq 3.5 \times 10^{3}$		

[a] For a description of strains, see Table 1.
[b] For classical strains, inocula were adjusted to 1×10^{6} to 2×10^{6}/ml (total dosage) at the ratios indicated, while El Tor strains were adjusted to 3×10^{5} to 8×10^{5}/ml (total dosage) at the ratios indicated.
[c] Competitive growth in LB broth overnight at 37°C.
[d] CFU recovered represent the total CFU in 5 ml of prepared intestinal extract ± standard deviations.

TABLE 6. Infant mouse colonization by type IV pilin mutants of *V. cholerae*

Competing strains[a]	Inoculum ratio[b]	In vivo competitive index[b] (no. of mice)
Classical O395 background		
KFV8 (Δ*pilA*) × Lac1 (Δ*lacZ*)	1.3	1.1 ± 0.1 (4)
KP9.79 (*tcp*) × Lac1 (Δ*LacZ*)	1.14	$<2 \times 10^{-6}$ (2)
El Tor N16961Sm background		
KFV6 (Δ*pilA*) × KFV10 (Δ*lacZ*)	1.17	0.49 ± 0.08 (7)
KFV11 (Δ*mshA*) × KFV10 (Δ*lacZ*)	1.28	0.78 ± 0.24 (6)
KFV12 (Δ*pilA* Δ*mshA*) × KFV10 (Δ*lacZ*)	1.57	0.76 ± 0.25 (5)
KFV33 (Δ*tcpA*) × KFV10 (Δ*lacZ*)	1.15	$<3.3 \times 10^{-6}$ (3)
Toxin-deficient background		
KFV9 (O395N1 Δ*pilA*) × KFV16 (O395N1 Δ*lacZ*)	0.37	1.4 ± 0.2 (4)
KFV38 (P4 Δ*pilA*) × KFV26 (P4 Δ*lacZ*)	2.1	0.55 ± 0.08 (3)

[a] For toxin-producing backgrounds, a 50-μl dose at 1×10^4 to 5×10^4/ml for each strain was delivered by oral gavage and processed as described in Materials and Methods. O395N1-based strains were given at a 10-fold higher inoculum, while P4-based strains were given at a 1,000-fold higher inoculum, to counteract reduced adherence by toxin-deficient strains.

[b] Expressed as CFU of *lacZ*$^+$ colonies/CFU of *lacZ* colonies ± standard deviations.

restored by *pilD* in *trans* (Fig. 6B). This result shows that the loss of adherence is most likely due to a gene or protein that was terminally mutated during reversion of the *pilD* mutation. Unfortunately, attempts to demonstrate an adherence defect in deletion strain KFV32 could not be interpreted because wild-type strains grown to a low density also failed to adhere (data not shown).

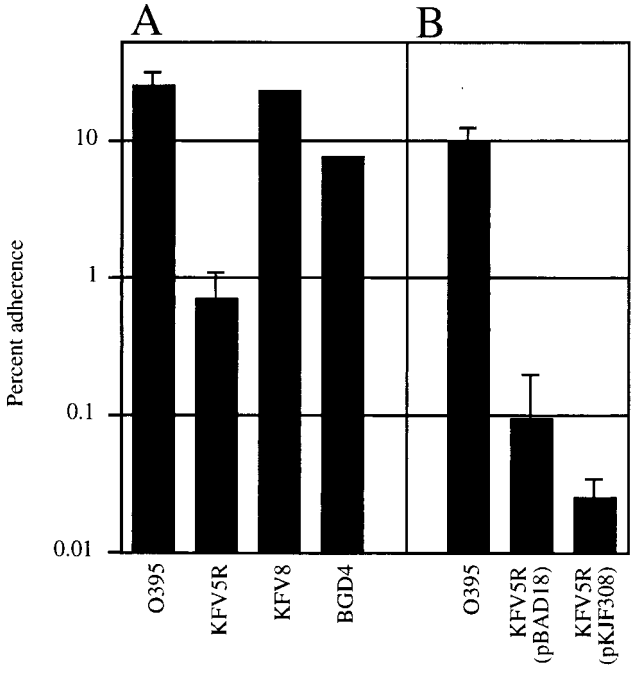

FIG. 6. Adherence of *V. cholerae* strains to HEp-2 cells. Assays were performed as described in Materials and Methods. KFV5R carrying either the pBAD18 vector or the *pilD*-complementing plasmid pKJF308 was grown in the presence of 0.05% arabinose to induce promoter P_{BAD}. Arabinose does not inhibit the adherence of wild-type O395 (data not shown). Results are presented as the percentage of input bacteria that adhered to HEp-2 cells after 1 h of incubation. Numbers given are averages from triplicate samples in a single assay and are representative of at least two experiments. Error bars represent standard deviations. (A) Adherence by O395 mutants of *V. cholerae*. (B) Complementation of KFV5R by *pilD* in *trans*.

Several genes are clear candidates for the adherence gene lost in the reversion process. The terminal mutation may have resulted from the loss of one or more of the prepilin proteins, since evidence suggests that the slow growth of *pilD* strains is due in part to the accumulation of unprocessed prepilin proteins (10). However, deletion of *pilA* or *tcpA* had little effect on adherence to HEp-2 cells (Fig. 6A). These data show that these two type IV pilin proteins (PilA and TcpA) do not function in epithelial cell adherence and that the terminal loss of *pilA* or *tcpA* could not have caused the defect in adherence observed for *pilD* mutant strains.

Another candidate for the lost protein is OmpU, an outer membrane protein previously proposed to be required for the adherence of *V. cholerae* to HEp-2 cells (48). Sandkvist et. al (44) demonstrated that disruption of the type II secretion machinery encoded by the *eps* genes has several pleiotropic effects, including the rapid degradation of OmpU. Since *pilD* mutants are also secretion machinery mutants, they may have a similar defect. Indeed, KFV5R and KFV32 had reduced expression of OmpU, but the expression of OmpU was restored to wild-type levels when *pilD* was introduced in *trans* (data not shown). Thus, if OmpU is solely responsible for the adherence defect, complemented KFV5R should adhere to HEp-2 cells. Therefore, the failure of KFV5R to adhere to HEp-2 cells must be due to another protein terminally mutated during the reversion process. Thus, the nature of the adherence defect in classical strains cannot be fully determined at this time, although it is likely to involve either a previously unrecognized PilD-processed protein or another outer membrane protein which is terminally lost in the reversion process.

The adherence of El Tor strains to HEp-2 cells is not *pilD* dependent. Determining the adherence of N16961Sm derivatives proved to be difficult, compared to that for the classical strains. We recently showed that *V. cholerae* El Tor and O139 strains produced a toxin that caused rounding and detachment of HEp-2 cells (30). The destruction of cellular integrity due to this toxin hampered interpretation of the results. Further, it was recently shown that MSHA pili mediated adherence to solid substrates, including laboratory plastic and glass items used for tissue culturing (58). To circumvent these technical difficulties, KFV18R with a disruption of the cytotoxin was compared to KFV11 (Δ*mshA*), which also had a disruption of the cytotoxin. Both strains showed 3% adherence to HEp-2 cells. This result shows that the loss of *pilD* and the subsequent reversion of growth defects to generate KFV18R did not permanently disrupt a key adhesin. However, the level of adherence was quite low compared to that observed for classical strains, suggesting that conditions favoring adherence by El Tor strains may be different from those for classical strains.

DISCUSSION

In recent years, the new field of functional genomics has expanded for prokaryotic research. It has been predicted that most of the important human bacterial pathogens will be completely sequenced by the year 2000 (52). The new challenge presented to researchers concerns the use of this plethora of information in designing and performing informative experiments. While sequencing of the *V. cholerae* genome is not yet complete, preliminary data were successfully used to identify and characterize a new type IV prepilin peptidase of *V. cholerae*, PilD. This prepilin peptidase is distinctly different from a previously described peptidase, TcpJ, showing only 25% iden-

tity at the amino acid level. This difference reflects the specificity for protein substrates. TcpJ appears to be responsible solely for processing of the type IV-B pilin protein TcpA (28), while PilD has a multitude of type IV-A substrates, including Eps proteins, essential for CT and HAP secretion, Msh proteins, essential for hemagglutination, and possibly TcpB, a protein essential for TCP production. In this study, we demonstrated that the loss of *pilD* disrupts each of these processes and that these defects are due solely to the loss of *pilD*, since the defects could be complemented in *trans*.

Recently, Marsh and Taylor (32) directly demonstrated that *V. cholerae pilD* (*vcpD*) encodes a type IV prepilin peptidase. They showed that EpsI and MshA produced in *E. coli* are N-terminally processed only when coexpressed with PilD. This observation confirms our prediction that secretion and MSHA defects are due to the lack of processing of type IV secretion signals by PilD.

Like us, Marsh and Taylor (32) showed that *pilD* (*vcpD*) mutants are defective for toxin secretion and MSHA, as well as for infant mouse colonization. However, they were unable to distinguish the phenotypes observed from the growth defects exhibited by the *pilD* (*vcpD*) mutants (32). In this work, our isolation and characterization of mutants with enhanced growth rates enabled us to characterize the role of *pilD* with less consideration for growth defects. These mutants were compared to more slowly growing deletion mutants, with similar results, and we further demonstrated that the defects were complemented by the introduction of *pilD* on a plasmid. Thus, we can firmly conclude that *pilD* is essential for secretion, MSHA, and TCP production.

In addition to the characterization of *pilD*, this work sheds light on continuing questions regarding the mechanism of bacterial adherence. Since type IV pili are essential for adherence in *P. aeruginosa* and *N. gonorrhoeae* (7, 16, 27, 43), it seems plausible that PilA is assembled into a new pilus essential for adherence and colonization. Surprisingly, *pilA* mutations resulted in no defects in these processes either alone or in combination with a mutation in *mshA*. Further, *pilA* mutants are not required for adherence to solid substrates, disputing a role for adherence in the environment (58). At present there is no information about the role of the putative pilus. However, the location of its gene within an operon with *pilD* indicates that it is expressed both in vivo and ex vivo. *pilD* is required for CT secretion and TCP production and thus must be expressed in vivo, and it is essential for MSHA, a phenotype expressed by bacteria grown in cultures. Indeed, preliminary analysis of *lacZ* fusions to the *pil* operon showed that the expression of this operon is initiated in the mid-log phase, just as *pilD* mutants begin to show severe growth defects (Fig. 3 and data not shown). Thus, it is unlikely that *pilA*, *pilB*, and *pilC* are silent genes coincidentally present in an operon with *pilD*. The 100% conservation of the *pilA* sequence between the classical and El Tor biotypes also indicates that *pilA* is likely to be important for some aspect of *V. cholerae* biology.

What, then, is the nature of the *V. cholerae* adherence factors mediating adherence to HEp-2 cells? One possible hint comes from the analysis of the classical mutant KFV5R. This mutant is a spontaneously arising second-site suppressor of the slow-growth defect in *pilD* kanπ insertion mutant KFV5. It has a defect in HEp-2 cell adherence that cannot be complemented in *trans* by the wild-type *pilD* gene (Fig. 6B). Thus, an adhesion factor must have been disrupted during the reversion process, allowing faster growth. This lost protein may be a type IV protein. It is clear that the loss of pilin subunits can lead to enhanced growth of *pilD* mutants of *N. gonorrhoeae* (10), and our ability to produce a *pilD* mutation only in the Δ*pilA* background of N16961Sm suggests that a similar mechanism may allow enhanced growth of *pilD* mutants of *V. cholerae*. Analysis of more recent genomic data obtained from The Institute for Genomic Research shows that at least five additional type IV cleavable proteins are encoded by the *V. cholerae* genome (56a). Loss of any of these five proteins may have enhanced the growth of the *pilD* mutant KFV5 while decreasing HEp-2 cell adherence. Systematic deletion of each of the five corresponding genes will determine if any of these proteins are essential for HEp-2 cell adherence.

The adherence factor lost may also be an outer membrane protein. Secretion mutants of *V. cholerae*, including *pilD* mutants, lose outer membrane proteins, particularly the putative adhesin OmpU (32, 44). However, the loss of OmpU probably does not cause the HEp-2 cell adherence defect in KFV5R, since the reversion process and complementation restored OmpU expression to KFV5R. Thus, another outer membrane protein may be essential for HEp-2 cell adherence. Sengupta et al. (45) showed that rabbit antisera raised against major outer membrane proteins are partially protective against *V. cholerae* challenge in infant mice. This observation argues that outer membrane proteins may play a role in intestinal colonization and adherence. Attempts to isolate outer membrane protein mutants by signature-tagged mutagenesis and in vivo colonization repeatedly identified genes required for TCP production (8). Thus, the nature of outer membrane proteins that may be essential for adherence could probably be identified only by screening of transposon mutants for defects in adherence in vitro. Such an approach has identified a 40-kDa outer membrane protein from *V. cholerae* O139 that shows decreased adherence and infant mouse colonization, although the disrupted gene has not yet been identified (5).

This work has demonstrated that genomic data coupled with experimentation can rapidly expand the understanding of bacterial pathogenesis. However, the successful identification of a second prepilin peptidase is juxtaposed to the inaccurate supposition that *pilA* encodes a major adherence factor. These observations highlight the importance of genetic experimentation for complementing genomic data to fully enrich the fields of prokaryotic biology and eukaryotic biology.

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