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Bundle-Forming Pilus Locus of *Aeromonas veronii* bv. Sobria

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Little is known about the colonization mechanisms of *Aeromonas* spp. Previous work has suggested that the type IV bundle-forming pilus (Bfp) is an aeromonad intestinal colonization factor. This study provides the first genetic characterization of this structure. To define the role of Bfp in *Aeromonas veronii* bv. Sobria adherence, a 22-kb locus encoding the bundle-forming pilus was isolated; this contained 17 pilus-related genes similar to the mannose-sensitive hemagglutinin (MSHA) of *Vibrio cholerae*. Reverse transcriptase PCR (RT-PCR) demonstrated that the locus had two major transcriptional units, *mshI* to *mshF* and *mshB* to *mshQ*. Transcriptional fusion experiments demonstrated the presence of two strong promoters upstream of *mshI* and *mshB*. The locus encoded four putative prepilin proteins, one of which (MshA) corresponded to the N-terminal sequence of the previously isolated major pilin protein. All the pilin genes were inactivated, mutation of each minor or major pilin gene greatly reduced the bacterium's ability to adhere and form biofilms, and complementation of each mutant in *trans* rescued this phenotype. Mutation of the major pilin MshA and MshB, a minor pilin, resulted in their loss. The position of the *mshH* gene is conserved within a number of bacteria, and we have shown it is not transcriptionally linked to the other *msh* genes; moreover, its mutation did not have a dramatic effect on either adhesion or biofilm formation. We conclude that the bundle-forming pilus is required for *A. veronii* bv. Sobria adherence and biofilm formation; furthermore, both the major and minor pilin proteins are essential for this process.

Mesophilic *Aeromonas* species are ubiquitous waterborne bacteria that are pathogens of reptiles, amphibians, and fish (1). They can be isolated as part of the fecal flora of a wide variety of other animals, including some used for human consumption, such as cows, sheep, and poultry. In humans, *Aeromonas hydrophila* belonging to hybridization groups 1 and 3 (HG1 and HG3), *Aeromonas veronii* bv. Sobria (HG8/HG10), and *Aeromonas caviae* (HG4) are the main species of the genus that have been associated with disease (9). *Aeromonas* spp. are an underreported cause of disease in humans and are regarded as emerging pathogens. In humans, they cause diseases ranging from serious wound infections to septicemia (20). However, most are isolated from cases of gastroenteritis that can range from a mild self-limiting diarrhea to a cholera-like illness to dysentery (32).

A range of putative virulence factors has been described for the aeromonads, ranging from the hemolytic toxin aerolysin and cytotonic toxins to capsules and extracellular enzymes (32), and recently aeromonad type III (T3SS) and type VI (T6SS) secretion systems have been described (29, 33).

Adhesion and colonization of host tissue are critical to aeromonad gastrointestinal pathogenesis to allow for effective delivery of toxins and/or invasion that results in disease. However, the adherence process of aeromonads is still poorly understood. A number of factors have been implicated in virulence, such as long/wavy (L/W) type IV pili, outer membrane proteins, lipopolysaccharide O antigen (O-Ag), and the polar flagellum (32). The mesophilic aeromonads are interesting, as most strains express two distinct flagellum systems (4, 22). They have a glycosylated polar flagellum for swimming in liquid and express separate lateral flagella for swarming over surfaces (4, 22, 30).

Type IV pili have been shown to be important for epithelial cell adherence and colonization for several pathogens, including enteric pathogens such as *Vibrio cholerae* and several types of pathogenic *Escherichia coli* (5, 27). Previous studies on gastroenteritis-associated *Aeromonas* spp. has shown that they also encode at least two distinct families of type IV pili, Tap and Bfp, which differ in their N-terminal amino acid sequences and molecular weights (2). The type IV *Aeromonas* pilus (Tap) was identified following the cloning of the biogenesis cluster (*tapABCD*) (21). Insertional inactivation of the structural pilin gene *tapA* had no effect on adherence, as the mutant cells were able to adhere to both biotic and abiotic surfaces as well as the wild-type parental strain (11). The predominant aeromonad type IV pilus that has been isolated is the bundle-forming pilus (Bfp), whose removal by mechanical shearing or blockage using anti-Bfp antibodies reduces the ability of aeromonads to adhere to host cells by over 80% (13, 14). The N-terminal amino acid sequence of Bfp shows closet homology to the mannose-sensitive hemagglutinin (MSHA) of *Vibrio cholerae* El Tor and that this type IV pilin belongs to the type IVa non-bundle-forming pilus family. Therefore, it appears that Bfp is the major pilus adhesin of mesophilic *Aeromonas* species, but they have not been genetically characterized. Moreover, the recently published genome sequences of *A. hydrophila* ATCC 7966^T (26) and *Aeromonas salmonicida* A449 (24) have shown that these strains encode three type IV systems, *tap*, *flp*, and *msh* (Bfp). However, in the psychrophilic species *A. salmonicida*, the locus encoding the MSHA pilus had a large internal deletion, and the pilus system was therefore thought to be nonfunctional (3). Mutation of the *A. salmonicida* Flp system made little or no contribution to virulence, but the Tap system was thought to be involved in virulence when tested in the natural host for this species, the Atlantic Salmon (3).

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TABLE 1 Bacterial strains and plasmids used in this study

Strain/plasmid	Genotype/phenotype[a]	Source/reference
Strains		
Aeromonas veronii bv. Sobria		
BC88	Wild type, dysenteric isolate	13
BC88R	BC88, spontaneous Rif^r	This study
BC88mshA	BC88R*mshA*::Km^r	This study
BC88mshB	BC88R*mshB*::Km^r	This study
BC88mshC	BC88R*mshC*::Km^r	This study
BC88mshD	BC88R*mshD*::Km^r	This study
BC88TapD	BC88R*mshD*::Km^r	This study
BC88MshH	BC88R*mshH*::Km^r	This study
Escherichia coli		
CC118λ*pir*	$\Delta(ara,leu)_{7697}$ $araD_{139}$ Δ*lazX74 glaE glaK phoA20 thi-1 rspE rpoB(Rf^r) argE(am) recA1* λ*pir*$^+$	8
S17-1λ*pir*	*hsdR pro recA*, RP4-2 in chromosome, Km::Tn*7* (Tc::Mu) λ*pir* Tp^r Sm^r	19
XL1-Blue	*endA1 recA1 hsdR17 supE44 thi-1 gyrA96 relA1 lac* [F′ *proAB lacI*^{q} *Z*ΔM15 Tn*10* (Tc^r)]	Stratagene
BL21(DE3)	F$^-$ *ompT gal dcm lon hsdS*$_B$ ($r_B{}^-$ $m_B{}^-$) λ(DE3 [*lacI lacUV5*-T7 gene 1 *ind1 sam7 nin5*])	Novagen
Staphylococcus epidermidis		
NCTC11047	Wild-type strain, laboratory culture collection	
Plasmid		
pUC19	High-copy-no. cloning vector, MCS, Amp^r	Gibco BRL
pUC4-KIXX	Source of Tn*5*-derived *nptII* gene (Km^r)	Pharmacia
pBBR1MCS	Broad host range, IncP, -W, -Q, ColE1 and p15A compatible, containing pBluescript IIKS-*lacZ*α-polylinker, Cm^r	15
pKNG101	*ori*R6K *mob*RK2 *strAB sacBR*, 6.8 kb, Sm^r	10
pZErO2.1	Cloning vector Km^r	Invitrogen
pET28a(+)	*E. coli* overexpression vector for N-terminal His_6-tagged proteins, Km^r	Novagen
pKAGb4(−)	Broad-host-range vector ori_{1600} carrying promoterless *lacZ* gene, Cm^r	(M. S. Thomas)
pTB037	78 bp, encoding MshA N-terminal region in pZErO2.1	This study
pETMshA	pET28a(+) expressing MshA as an N-terminal His_6-tagged protein, Km^r	This study
pETMshB	pET28a(+) expressing MshB as an N-terminal His_6-tagged protein, Km^r	This study
pBBRMshA	*mshA* of *A. veronii* bv. Sobria BC88 in pBBR1MCS, Cm^r	This study
pBBRMshB	*mshB* of *A. veronii* bv. Sobria BC88 in pBBR1MCS, Cm^r	This study
pBBRMshC	*mshC* of *A. veronii* bv. Sobria BC88 in pBBR1MCS, Cm^r	This study
pBBRMshD	*mshD* of *A. veronii* bv. Sobria BC88 in pBBR1MCS, Cm^r	This study
pBBRMshH	*mshH* of *A. veronii* bv. Sobria BC88 in pBBR1MCS, Cm^r	This study
pBBRTapD	*tapD* of *A. veronii* bv. Sobria BC88 in pBBR1MCS, Cm^r	This study
pKAG-PB	Promoter probe suicide vector pKAGb4 with BC88 *mshB* promoter region ligated upstream of the promoterless *lacZ* gene, Cm^r	This study
pKAG-PA	Promoter probe suicide vector pKAGb4 with BC88 *mshA* promoter region ligated upstream of the promoterless *lacZ* gene, Cm^r	This study
pKAG-PC	Promoter probe suicide vector pKAGb4 with BC88 *mshC* promoter region ligated upstream of the promoterless *lacZ* gene, Cm^r	This study
pKAG-PD	Promoter probe suicide vector pKAGb4 with BC88 *mshD* promoter region ligated upstream of the promoterless *lacZ* gene, Cm^r	This study
pKAG-PI	Promoter probe suicide vector pKAGb4 with BC88 *mshI* promoter region ligated upstream of the promoterless *lacZ* gene, Cm^r	This study

[a] Amp, ampicillin; Cm, chloramphenicol; Km, kanamycin; Nal, nalidixic acid; Sm, streptomycin; MCS, multiple cloning site.

In this study, we present the first data on the genetic characterization of the bundle-forming pilus of mesophilic aeromonads and demonstrate that it has a role in the colonization of both biotic and abiotic surfaces.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids are listed in Table 1. Bacteria were grown aerobically overnight (16 to 20 h), either statically or by shaking, at 37°C. They were grown either in brain heart infusion broth (BHIB) (Oxoid) or on Luria-Bertani agar (LBA), supplemented with the appropriate antibiotics when required. Working stocks of the strains were kept on LBA plates at 4°C for a maximum of 2 weeks. Rifampin (Rif), ampicillin (Amp), streptomycin (Sm), and kanamycin (Km) were used at final concentrations of 50 μg/ml, whereas chloramphenicol (Cm) and tetracycline (Tc) were used at 25 μg/ml.

Protease, DNase, and hemolysin detection. For protease plates, the method described by Sokol et al. (28) was used. Dialyzed brain heart infusion (D-BHI) milk medium was prepared by dissolving 18.5 g of BHI powder (Difco) in 50 ml of water that was then dialyzed against 1 liter of distilled water (dH_2O) for 18 h at 4°C. Agar (Difco) was added to the dialysate to a concentration of 3% (wt/vol). A 3% (wt/vol) solution of skim milk was prepared, and the solutions were autoclaved separately. Equal volumes of the two sterile solutions were mixed at 60°C, and the mixture was dispensed into petri plates. The bacterial strains were cultured on the plate and incubated at 37°C for 24 h. The presence of clear

zones of hydrolysis on the D-BHI milk medium was considered a positive result.

For DNase detection, appropriate *Aeromonas* strains in addition to a *Staphylococcus epidermidis* wild-type strain were grown on a DNase agar plate (Oxoid) at 37°C overnight for approximately 16 h. The plate was then flooded with 1 M HCl to precipitate the DNA and make the plate opaque. A positive reaction was indicated by the disappearance of the opaqueness and an area of clearing around the colony of bacterial growth. For hemolysin detection, *Aeromonas* strains were streaked onto a Columbia blood agar plate containing horse blood (Oxoid). Plates were incubated at 37°C overnight for approximately 16 h and examined for β-hemolysis.

Pilin purification. The pilins MshA and MshB were overexpressed and purified as N-terminal His_6-tagged proteins. Both *mshA* and *mshB*, encoding the corresponding proteins minus their signal sequences, were amplified using primers, including NdeI/XhoI and NdeI/EcoRI restriction sites, respectively, to facilitate cloning in frame into the overexpression vector pET28a (Novagen). The proteins were overexpressed in *E. coli* BL21λ(DE3) according to the manufacturer's instructions and then purified using a HisTrap HP column (GE Healthcare).

Preparation of antibody. Approximately 200 μg of purified pilin was emulsified with 1 ml of Freund complete adjuvant and inoculated subcutaneously into dwarf lop-eared rabbits. Booster injections of the 100-μg pilin protein in 0.5 ml Freund complete adjuvant were administered 4 and 6 weeks later. Antibodies were obtained by bleeding 10 days after the second booster injection.

Whole-cell protein preparation, SDS-PAGE, and immunoblotting. *Aeromonas* strains were grown statically overnight in BHIB at 37°C. Equivalent numbers of cells were harvested by centrifugation, and the cell pellet was either boiled in SDS-PAGE loading buffer for whole-cell proteins or was resuspended in 1 ml of phosphate-buffered saline (PBS), which was subsequently sheared of pili by passing the bacterial suspension through an 18-gauge needle 20 times. Bacteria were pelleted by centrifugation and discarded; 100 μl of the supernatant was added to 100 μl of SDS-PAGE loading buffer and boiled for 5 min. Protein samples were separated on SDS-polyacrylamide gels (12% [vol/vol]). For immunoblotting, proteins were transferred onto a Hybond-C (GE Healthcare) nitrocellulose membrane. Following transfer, membranes were blocked with 5% (wt/vol) powdered skim milk and probed with a polyclonal rabbit anti-pilin antibody (1:500). The unbound antibody was removed by five washes in PBS, and a goat anti-rabbit peroxidase-conjugated secondary antibody (1:1,000) was added. The unbound secondary antibody was washed away with PBS as described for the primary antibody. The conjugate was then detected using the ECL detection system (GE Healthcare).

Adherence assay. Tissue culture was maintained as described by Thornley et al. (31). The HEp-2 cells (ATCC CCL23, derived from human epidermoid larynx carcinoma) were grown in basal Eagles medium containing newborn calf serum. The cell line was grown until confluent at 37°C in air with 5% CO_2, diluted to 50 to 100 cells/mm^2, and seeded in 1-ml amounts into 24-well tissue culture plates. The HEp-2 cells were used at semiconfluence in duplicate for each separate experiment, with monolayer age being kept as constant as possible. The HEp-2 cells were 24 h old after the final seeding. Bacteria were grown statically in BHIB at 37°C, harvested by gentle centrifugation (1,600 × *g*, 5 min), and resuspended in PBS (pH 7.2) at approximately 10^7 CFU/ml (A_{600} of ~0.07). The HEp-2 cells were infected with 1 ml of the bacterial suspension for 90 min at 37°C in 5% CO_2. Following infection, the nonadherent bacteria were removed from the monolayer by three washes with PBS. The remaining adherent bacteria and the monolayers were then fixed in 100% methanol for 5 min. Methanol was removed by washing with PBS, and the HEp-2 cells with the adherent bacteria were stained for 45 min in 10% (vol/vol) Giemsa stain (BDH, United Kingdom) prepared in Giemsa buffer. The coverslips were air dried, mounted, and viewed by oil immersion under a light microscope at ×1,000 magnification. Twenty HEp-2 cells/coverslip were randomly chosen, and the number of bacteria adhering/HEp-2 cell was recorded. Assays were carried out in duplicates or triplicates.

Biofilm assay. Biofilm formation in borosilicate glass tubes (10 by 75 mm) was assessed by the method of O'Toole and colleagues with slight modifications (12). In brief, the glass tubes were inoculated with 300 μl of a 1:100 dilution of bacteria from brain-heart infusion broth (BHIB) cultures grown overnight (16 to 18 h). These were then lightly covered with foil and incubated for up to 30 h at 37°C without shaking. To detect and quantify biofilm formation, the tubes were rinsed thoroughly and vigorously with water, and the remaining cells were stained with crystal violet solution (0.5% [wt/vol], 15 min at room temperature [RT]). The crystal violet-stained biofilm was then solubilized by the addition of 100% ethanol (600 μl, 10 min, RT), and the optical density at 570 nm (OD_{570}) of 200 μl of the resultant suspension was measured using a microplate reader (Bio-Rad, Hercules, CA). Replicates of three to six tubes per organism were examined in each experiment, and a broth control that was not inoculated was included in each experiment. The growth rates and motility of the pilus mutant strains were assessed by measuring OD_{600} and swimming in motility agar (22), respectively, and were shown not to differ significantly from that of the wild-type strain (data not shown).

Statistical analysis. The differences in adherence to cell lines between the wild-type and mutant strains and the mutant strains versus the complemented strains were analyzed using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA). Data are given as means ± standard deviations (SD). Statistical significance was compared to the wild-type by one-way analysis of variance (ANOVA).

DNA techniques. Plasmid DNA was isolated by alkaline lysis or by using the Qiagen miniprep DNA purification system (Qiagen). *A. veronii* bv. Sobria chromosomal DNA isolation was carried out according to standard techniques (25). DNA restriction digestions and T4 ligations were carried out according to the manufacturer's instructions. DNA samples were separated on 0.8% (wt/vol) agarose gels; when required, extraction of DNA from gels was carried out using the QIAquick gel extraction kit (Qiagen).

PCR. Reactions were performed using *Pfx* DNA polymerase (Invitrogen) at 2.5 mM $MgCl_2$ in a Techne thermal cycler. Initial DNA denaturation was carried out for 2 min, and amplification reactions were carried out for 30 cycles with denaturation at 95°C for 30 s, primer annealing at 55°C for 1 min, and elongation at 72°C for 2 min.

Southern and dot blot hybridizations. Southern and dot blot hybridizations were performed by capillary transfer (25). Probe labeling, hybridization, and detection were carried out using the enhanced chemiluminescence (ECL) labeling and detection system (GE Healthcare) according to the manufacturer's instructions.

Nucleotide sequencing and sequence analysis. Double-stranded DNA sequencing was performed by using the Sanger dideoxy-chain termination method with the Abi Prism dye terminator cycle sequencing kit (Perkin Elmer company). DNA fragments were ligated into pUC19 and sequenced using an ABI Prism 377 DNA sequencer (Perkin Elmer company). The M13 universal primers were employed in sequencing the ends of the DNA inserts. Following the first sequencing reaction and whenever required, primers were designed until the insert sequences were complete. Primers used for DNA sequencing were purchased from Eurogentec. For chromosomal walking to extend the sequence into flanking regions, direct genomic sequencing was used. Custom 24-mer primers were designed to a known nucleotide sequence and were used with sheared *A. veronii* bv. Sobria genomic DNA in a 99-cycle polymerase reaction using the BigDye Terminator mix according to the manufacturer's instructions (Perkin Elmer company). The DNA sequence was translated in all six frames, and all open reading frames (ORFs) greater than 100 bp were inspected. Deduced amino acid sequences were compared with those of DNA translated in all six frames from nonredundant GenBank and EMBL databases by using the BLAST network service at the National Center for Biotechnology Information and analyzed as previously described (6, 22).

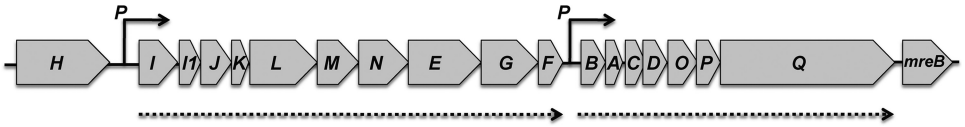

FIG 1 Genetic organization of the *A. veronii* bv. Sobria BC88, mannose-sensitive hemagglutinin (MSHA) type IV pilus locus. The predicted ORFs were named after the *msh* homologues in other species and are indicated by gray arrows, which indicate the direction of transcription. The right-angled arrows with solid lines and a *P* depict the promoter regions, whereas the arrows with dotted lines show the linked transcriptional units.

Construction of defined insertion mutants. Mutants were created by the insertion of the Tn*5*-derived kanamycin resistance cartridge (*npt*II) from pUC4-KIXX (Pharmacia). This cartridge contains an outward reading promoter that drives the transcription of downstream genes when inserted in the correct orientation. For each mutant, the 1.4-kb SmaI-digested kanamycin resistance cartridge was inserted into a convenient restriction site within the middle of the gene. If a convenient site was not present, one was created by spliced overlap extension (SOE) PCR. Constructs containing the mutated genes were ligated into the suicide vector pKNG101 (10) and transferred into *Aeromonas* by conjugation. Conjugal transfer of the recombinant plasmids from *E. coli* S17-1λ*pir* to *A. veronii* bv. Sobria BC88 was performed using a filter mating technique. Bacterial conjugation was allowed to proceed for 6 to 8 h at 37°C on sterile nitrocellulose filters (0.45-mm pore size) placed onto an LBA plate. Serial dilutions of the mating mix were then plated on LBA supplemented with Rif and Km, the latter added in order to select for recombination. Colonies that were kanamycin resistant (Km^r) and streptomycin sensitive for pKNG101 derivatives (those not likely to have retained the vector) were purified and probed for the kanamycin cartridge and absence of any plasmid sequences by Southern hybridization, thus demonstrating a double recombination event and allelic exchange.

Construction of *lacZ* transcriptional fusions. The mobilizable broad-host-range *lacZ* promoter probe plasmid pKAGb-4(−) was used in this study (K. Agnoli and M. Thomas, unpublished data). The putative promoter regions of the four *A. veronii* bv. Sobria pilus genes and *mshI* gene were amplified by PCR, and the resulting fragments were directionally ligated separately into pKAGb-4(−), giving the plasmids pKAG-PB, pKAG-PA, pKAG-PC, pKAG-PD, and pKAG-PI for the *mshBACD* promoters and the *mshI* promoter, respectively. These plasmids were introduced separately by conjugation into the wild-type strain. Activity of the four putative promoters was measured as a function of β-galactosidase activity. *A. veronii* bv. Sobria cultures were grown in triplicate to an optical density at 600 nm (OD_{600}) of 0.5 to 0.8 and were then chilled on ice for 15 min. Duplicate assays were performed at 30°C on 200 μl of cells for each culture in a total volume of 1 ml following permeabilization of the cells with chloroform and sodium-dodecyl sulfate; values are presented in Miller units (MU) (18).

RT-PCR. Reverse transcriptase PCR (RT-PCR) was performed as described by Tabei et al. (30).

Nucleotide sequence accession number. The nucleotide sequence of the genes described here have been assigned the following GenBank accession number: JQ361815.

RESULTS

Cloning of the *bfp* pilus locus. At the outset of this project, the genome sequence of *Aeromonas veronii* bv. Sobria was not available. The N-terminal sequence of the major pilin purified from *A. veronii* bv. Sobria strain BC88 (13) was used to design the degenerate forward and reverse primers P019 (ATGACIYTIATHGARYTIGT) and P022 (TTIARRAAYTTIGGIGCIGC) that corresponded to amino acids 1 to 7 and 26 to 20, respectively. This enabled the amplification of a 78-bp DNA fragment by PCR, which was subsequently cloned into pZErO2.1, generating pTB037. Upon nucleotide sequencing, the translated sequence of the insert exactly corresponded to the N-terminal sequence of the Bfp pilin protein. The DNA sequence both upstream and downstream of this locus was then determined by direct genomic sequencing of the BC88 chromosomal DNA using custom-designed primers. This strategy extended the sequence by over 20 kb.

Sequence analysis revealed 19 ORFs (Fig. 1A, Table 2). The

TABLE 2 Characteristics of the *A. veronii* bv. sobria BC88 *msh* locus-encoded proteins

msh locus gene product	Mol mass (kDa)	Similarity to equivalent *V.cholerae* El Tor N16961 MSHA protein	Predicted function
MshH	74.2	5.6e−104	GGDEF/EAL domain-containing regulatory protein
MshI	31.3	4.9e−25	Bitopic inner membrane protein, GspL-like N-terminal domain of MshI
MshI1	22.3	6.2e−23	Bitopic inner membrane protein, GspL-like C-terminal domain of MshI
MshJ	24.3	3.4e−26	Bitopic inner membrane protein, GspM-like
MshK	11.4	3.13e−09	Periplasmic protein, GspC-like
MshL	59.9	1.4e−125	Outer membrane secretin, GspD-like
MshM	33.6	2.7e−49	Membrane protein with ATPase domain, GspA-like
MshN	39.5	2.1e−23	Tetratricopeptide repeat protein, PilF-like
MshE	63.2	9.0e−161	ATPase, GspE-like
MshG	44.9	7.3e−111	Polytopic integral membrane protein
MshB	22.4	5.7e−24	Prepilin
MshA	14.3	0.77	Prepilin
MshC	14.7	9.1e−7	Prepilin
MshD	15.4	7.6e−13	Prepilin
MshO	16.1	4.6e−8	Pseudopilin, GspG-like
MshP	16.7	7.4e−15	Periplasmic type IV pilus protein PilX
MshQ	144.5	5.0e−144	Pilin adhesin, PilY1-like, lectin-like domain

TABLE 3 β-Galactosidase activities of putative *msh* promoters in *A. veronii* bv. Sobria BC88

Plasmid	Construct	Activity (MU)
pKAGb4	Vector	300 ± 54
pKAG-PB	*mshBp-lacZ*	6,082 ± 564
pKAG-PA	*mshAp-lacZ*	212 ± 45
pKAG-PC	*mshCp-lacZ*	233 ± 44
pKAG-PD	*mshDp-lacZ*	233 ± 45
pKAG-PI	*mshIp-lacZ*	8,415 ± 653

arrangement and encoded protein sequences of the ORFs in this region closely resemble those of the type IV pilus mannose-sensitive hemagglutinin (MSHA) proteins of *V. cholerae* El Tor (17) and have been designated *mshA* to *mshQ*, accordingly. The locus encoded four pilin/pseudopilin proteins and 14 proteins that have been linked with pilus biogenesis in other bacteria; in addition, there was a hypothetical protein (MshF) that is encoded at the same position in *A. hydrophila* ATCC 7966^T and a diguanylate cyclase and phosphodiesterase domain-containing protein (MshH). Downstream of the locus was the gene *mreB* that encodes a protein involved in the determination of cell shape. Recently, the genome sequences of the related *Aeromonas* species, *A. hydrophila* ATCC 7966^T, *A. salmonicida* A449, and *A. veronii* B565, have been completed (16, 24, 26), and these also contained the 22-kb *msh* locus, with the analogous genes designated AHA0383 to AHA0399, ASA3949 to AHA3938, and B565_3672 to B565_3654, respectively. Moreover, the corresponding gene cluster is present in many *Vibrio*, *Photobacterium*, and *Shewanella* strains. All 18 genes that are thought to be required for the biogenesis of the bundle-forming pilus are present in *A. hydrophila*, but *A. salmonicida* A449 has a large deletion between the *mshN* and *mshD* genes (3). Unusually for MSHA, the *A. veronii* bv. Sobria MshI protein was encoded by two genes, *mshI* and *mshI1*. This was also true in *A. hydrophila* ATCC 7966^T (26), *A. salmonicida* (24), and *A. caviae* Sch3 (J. Shaw, unpublished data).

Transcript mapping of the pilus locus. RT-PCR was employed to determine which genes were cotranscribed with their immediate partners downstream. Primer pairs that overlapped different pilus genes (from *mshH* to *mreB*) within the locus were designed toward the 3′ end of the upstream gene and near the 5′ end of the downstream gene. This was in order to amplify the intergenic region between the two genes that would be expressed if the two genes were cotranscribed. RT-PCR products of the expected sizes were detected (data not shown) for every gene pair from *mshI* to *mshF* and from *mshB* to *mshQ*. This suggested the presence of two major transcriptional units, one incorporating *mshI* to *mshF* and the other *mshB* to *mshQ* in the pilus locus, with the two major promoters being located upstream of *mshI* and *mshB*, respectively (Fig. 1). It also suggested that both *mshH* and *mreB* were not transcriptionally linked to the other *msh* genes.

The four pilin genes (*mshBACD*) were located adjacent to each other in the same transcriptional orientation, with relatively small intergenic regions between the end of one gene and the start of another. These data along with the RT-PCR information suggested that they were part of a polycistronic operon. To support this, the putative promoter regions of the pilin genes *mshB*, *mshA*, *mshC*, and *mshD* were transcriptionally fused to the promoterless reporter gene *lacZ* in pKAGb-4(−). DNA fragments of around 500 bp were amplified by PCR from BC88 wild-type strain chromosomal DNA. These represented the 5′ ends of the genes and their putative promoter regions. The PCR fragments were ligated into the broad-host-range *lacZ* expression vector pKAGb-4(−) in an orientation to allow expression of the *lacZ* gene. The constructs were separately introduced into the *A. veronii* bv. Sobria wild-type strain BC88. Quantitative assays on cells grown in BHI for β-galactosidase activity showed that in the *A. veronii* bv. Sobria wild-type parental strain, the putative *mshA*, *mshC*, and *mshD* promoters had very low intrinsic β-galactosidase activities equivalent to that of the vector-only background control (212 to 300 MU). In contrast, a 20-fold increase in β-galactosidase activity (6,082 MU) was detected for the *mshB* promoter (Table 3), suggesting that the *mshBACD* genes comprise a single transcriptional unit.

Similarly, the *mshII1JKLMEGF* genes are located adjacent to each other in the same transcriptional orientation and appear to be part of a single transcriptional unit under the control of a promoter upstream of *mshI*. Therefore, the 500-bp region upstream of *mshI* was also amplified by PCR and ligated upstream of *lacZ* in pKAGb-4(−). When introduced into *A. veronii* bv. Sobria wild-type cells, this promoter repeatedly showed a higher activity (8,415 MU) than the *mshB* promoter (Table 3), suggesting that the expression of *mshII1JKLMEGF* is controlled by a promoter upstream of *mshI*.

The *A. veronii* bv. Sobria *msh* pilin genes are critical for adherence to epithelial cells. The *msh* locus contained four putative pilin/pseudopilin genes. As stated above, the N-terminal sequence of the purified pilin exactly matched that of the derived N terminus (minus cleaved signal sequence) of the *mshA* gene product (13). The major aim of this study was to create isogenic mutants in the major and minor pilin genes that would allow the first determination of the aeromonad bundle-forming pilus in adhesion. A kanamycin resistance cassette was inserted in the same transcriptional orientation as the target gene. The presence of an outward reading promoter on the cassette ensures expression of downstream genes, thereby reducing any polar effects. However, such insertions may alter the regulation of the downstream genes. The construction of all mutants was verified by Southern hybridization of chromosomal DNA and by PCR (data not shown).

Furthermore, both the MshA and MshB proteins as examples of both major and minor pilins were overexpressed as N-terminal His-tagged proteins in *E. coli* and purified. They were subsequently used to raise polyclonal antibodies against MshA and MshB in rabbits. Western blotting demonstrated that the MshA or MshB pilins were absent from their respective mutants, *mshA* and *mshB*, but present in the wild-type *mshC*, *mshD*, and *mshH* mutants (Fig. 2A and B). A mutant in the prepilin N-peptidase TapD was also created (see section below), and both pilins (MshA and MshB) were absent in the TapD mutant (Fig. 2A and B).

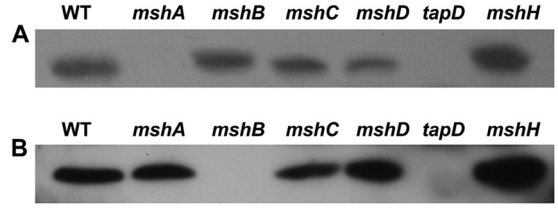

FIG 2 Msh-pilin immunoblotting of whole-cell proteins of the wild-type strain (WT) and isogenic mutants. The mutation is indicated above the lane. (A) MshA Western immunoblot; (B) MshB Western immunoblot. Proteins were obtained from bacteria grown at 37°C in BHIB and were analyzed by SDS-PAGE (12%). They were transferred onto nitrocellulose membranes and immunoblotted with anti-MshA or anti-MshB antibody (1:1,000).

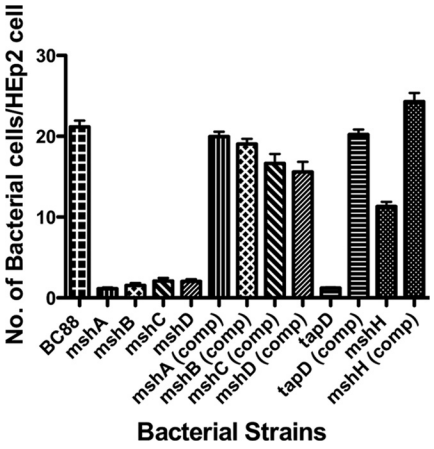

FIG 3 Adherence of *A. veronii* bv. Sobria strains to HEp-2 cells. Bacteria were grown statically in BHIB at 37°C, and adherence assays were carried out. Assays were carried out in triplicate on three separate occasions, and the mean number of adherent bacteria per HEp-2 cell was recorded. The error bars represent one standard deviation. Strains are indicated under the *x* axis, and significance of the mutants versus the wild-type and the complemented strains was by ANOVA ($P < 0.001$).

To determine if the *msh* locus encoding the *A. veronii* bv. Sobria BC88 bundle-forming pilus had a true role in adhesion, the wild-type and mutant strains were assessed by adherence to HEp-2 cells and by biofilm formation on borosilicate tubes. The wild-type strain BC88 adheres to HEp-2 cells with approximately 21 bacteria per cell, whereas in the mutants where each pilin gene had been individually knocked out, bacterial adherence was significantly ($P < 0.001$) reduced by over 90% to between 1 to 2 bacteria per cell (Fig. 3). To confirm these results and rule out any polar effects, complementation analysis was performed. Each of the individual pilin genes was amplified by PCR and cloned into the broad-host-range vector pBBR1MCS in an orientation to allow expression from the *lac* promoter. When such vectors were mobilized into the corresponding mutant strains, the adherence phenotype was rescued and near-wild-type levels of adhesion were observed ($P < 0.001$) (Fig. 3).

The *A. veronii* bv. Sobria pilin genes are essential for biofilm formation. In previous studies investigating the role of *A. caviae* flagella in biofilm formation, borosilicate glass gave more consistent results (12). Here, we used the same assay to investigate the role of the pilin mutants in biofilm formation over time (Fig. 4). Mutation of any of the pilin genes resulted in a dramatic reduction in the ability to form a biofilm after 12 h of incubation (Fig. 4A). At this time point, biofilm absorbance values were 10 to 20% of the wild-type values. At 24 h, biofilm formation by the mutant strains was again approximately 10% of that of the wild type (Fig. 4B), and by 48 and 72 h the mutant biofilm was between 30 and 50% of the wild type (Fig. 4C and D). Reductions in biofilm formation in the mutants at all time points were significant ($P < 0.001$). Complementation with the individual wild-type pilin genes expressed

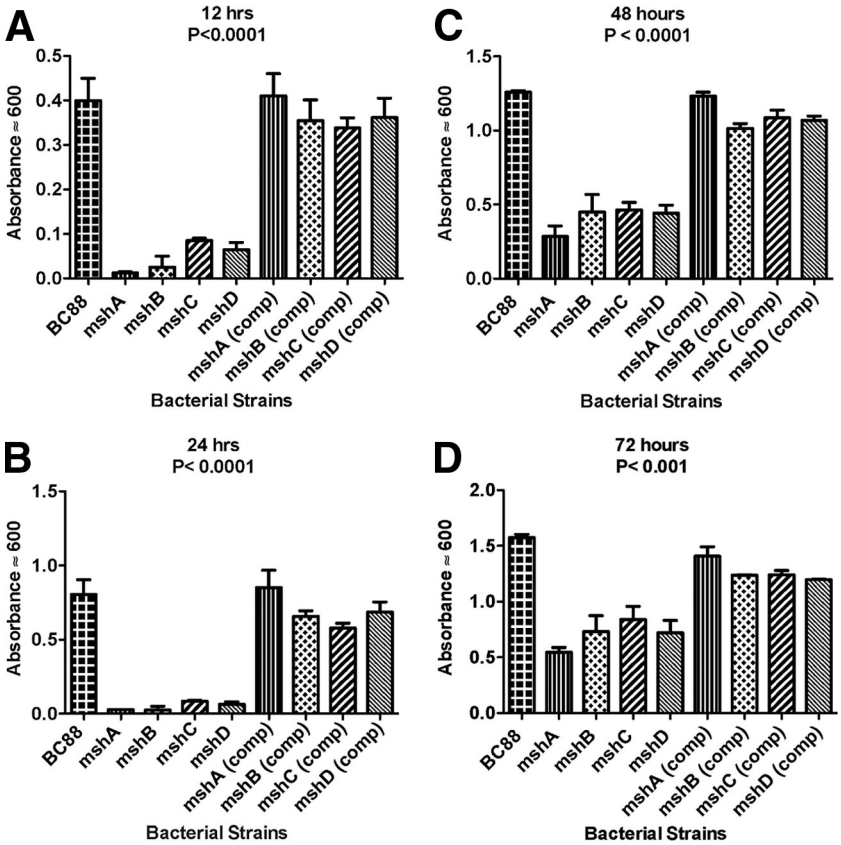

FIG 4 Biofilm development on borosilicate glass (37°C) over time by the *A. veronii* bv. Sobria wild-type, Msh-pilin mutant, and complemented strains. Values shown are the means of results from three replicate tubes ± standard deviations, and significance of the mutants versus the wild-type and complemented strains was by ANOVA ($P < 0.001$).

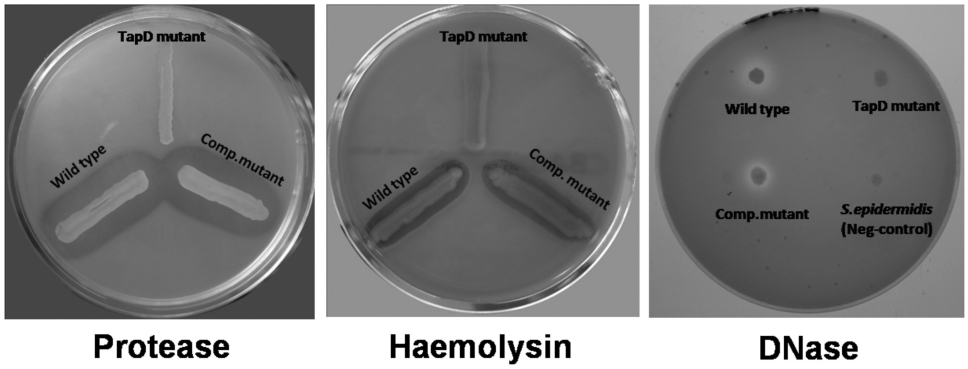

FIG 5 Analysis of secreted enzyme production of *A. veronii* bv. Sobria wild-type, *tapD* mutant, and complemented *tapD* mutant strains. Bacterial strains were plated onto protease agar, blood agar, or DNase agar plates, and a positive reaction was assessed due to the zones of clearing around the areas of growth.

in pBBR1MCS rescued the ability to form biofilms; this was especially obvious at 12 and 24 h ($P < 0.001$) (Fig. 4A and B).

The TapD prepilin peptidase is essential for epithelial cell adherence and biofilm formation. In each of the published aeromonad genome sequences, there is only one copy of the prepilin peptidase gene (*tapD*) that is located in the *tapABCD* locus that encodes part of another type IV pilus system (11). TapD is required for the processing and removal of the signal sequences from both pilins and pseudopilins. Therefore, TapD should be required not only for the assembly and functionality of the Tap type IV pilus but also for the MSHA (Bfp) type IV pilus and the type II secretion system (T2SS). Its mutation should also affect the adhesion of all *A. veronii* bv. Sobria type IV pilus systems. The *tapABCD* gene cluster has been previously isolated and sequenced from *A. veronii* bv. Sobria BC88 (11). Therefore, *tapD* was amplified by PCR and mutated using the method employed for the pilin genes, and its phenotype was investigated. To confirm the phenotype of the *tapD* mutant, its ability to secrete DNase, protease, and hemolysin was determined. As expected, the *tapD* mutant was negative for DNase production on DNase plates, negative for hemolysin production, and negative for protease production (Fig. 5). The wild-type copy of the *tapD* gene was amplified by PCR and cloned into the broad-host-range vector pBBR1MCS in an orientation to allow expression from the *lac* promoter. When this construct was mobilized into the corresponding *tapD* mutant strain for complementation analysis, the secreted enzyme production was restored (Fig. 5).

Based on the absence of another potential prepilin peptidase (26), we predicted that TapD would be required for the production of both the Msh and Tap type IV pilus systems, and its mutation resulted in a significant reduction of adherence ($P < 0.001$), with around 1 bacterium per tissue culture cell (Fig. 3). Mutation of TapD also had a great effect on biofilm formation, resulting in approximately 20% of that of the wild type (Fig. 6A). Complementation restored both adherence levels and biofilm formation to around those of the wild type ($P < 0.001$) (Fig. 3, Fig. 6A).

Role of MshH in epithelial cell adherence and biofilm formation. Upstream of *mshI* is a gene, *mshH*, that is conserved in the MSHA loci of a number of bacteria, including *Photobacterium*, *Shewanella* spp., and *Vibrio* spp., and has been annotated as an MSHA biogenesis gene. However, Marsh and Taylor (17) and work in this report by RT-PCR showed that this gene was not coupled or transcribed with other genes in the *msh* locus. We therefore aimed to determine if mutation of *mshH* would result in a phenotype similar to those observed for the other *msh* genes with regard to adhesion and biofilm formation. The *mshH* mutant strain showed a 48% reduction in adhesion to HEp-2 cells compared to that of the wild-type strain, resulting in approximately 11 bacteria per HEp-2 cell versus over 21 bacteria per cell ($P < 0.001$) (Fig. 3). Complementation using a wild-type copy of *mshH* in pBBR1MCS rescued the adherence phenotype to wild-type levels (Fig. 3). However, mutation of *mshH* did not appear to have a great effect on biofilm formation, as the levels of the mutant were close to those of the wild type at all time points, and any large decreases that had been observed for the pilin or *tapD* mutants were absent (Fig. 6B).

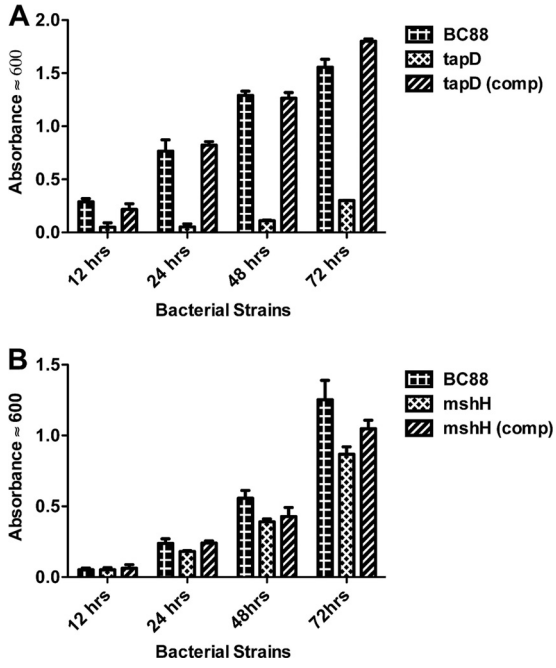

FIG 6 Biofilm development on borosilicate glass (37°C) over time by the *A. veronii* bv. Sobria wild-type, the TapD mutant, and complemented strains (A) and the MshH mutant and complemented strains (B). Values shown are the means of results from three replicate tubes ± standard deviations, and significance of the mutants versus the wild-type and the complemented strains was by ANOVA ($P < 0.001$).

TABLE 4 Sources of reference and test strains tested for hybridization with the 3′*mshB-mshA*-5′*mshC* probe

Strain	Source	*Aeruginosa* species	Hybridization
BC88	Stool	*A. veronii* bv. Sobria	+
ATCC 7966^T	Canned milk	*A. hydrophila* (HG1)	+
CDC 9533-76	Fish	*A. bestiarum* (HG2)	+
CDC 0862-83	Freshwater	*A. salmonicida* (HG3)	−
ATCC 15468^T	Guinea pig	*A. caviae* (HG4)	+
CDC 0862-83	Fish	*A. media* (HG5A)	+
ATCC 23309	Freshwater	*A. eucrenophila* (HG6)	+
CIP 7433^T	Fish	*A. sobria* (HG7)	+
CDC 0437-80	Stool	*A. veronii* bv. Sobria (HG8)	+
CDC 0787-80	Stool	*A. jandaei* (HG9)	+
ATCC 35624^T	Sputum	*A. veronii* bv. Veronii (HG10)	+
AH-1	Fish	*A. hydrophila*	+
AH-3	Fish	*A. hydrophila*	+
Sch13	Stool	*A. hydrophila*	+
Sch27	Stool	*A. hydrophila*	+
AH/V101	Stool	*A .hydrophila*	+
BC96	Stool	*A. veronii* bv. Sobria	+
FA132	Stool	*A. veronii* bv. Sobria	+
BC96	Stool	*A. veronii* bv. Sobria	+
CA110	Stool	*A. veronii* bv. Sobria	+
Sch22	Stool	*A. veronii* bv. Sobria	+
Sch39	Stool	*A. veronii* bv. Sobria	+
F 1965	Stool	*A. veronii* bv. Sobria	+
Sch3	Stool	*A. caviae*	+
Sch18	Stool	*A. caviae*	+
Sch29	Stool	*A. caviae*	+
Sch46	Stool	*A. caviae*	+
CA195	Stool	*A. caviae*	+
NCMB 1102	Fish	*A. salmonicida*	−
NB8601	Fish	*A. salmonicida*	−
MT1326	Fish	*A. salmonicida*	−

Prevalence of the *msh* locus among the aeromonads. Using a 1-kb PCR-generated probe that corresponded to the 3′ end of the *mshB* gene, the 5′ end of the *mshC* gene, and the complete *mshA* gene, we investigated the distribution of the *msh* locus among 31 aeromonads by Southern hybridization. The strains investigated included *A. hydrophila*, *A. veronii* bv. Sobria, *A. caviae*, *A. salmonicida*, and several type strains for the aeromonad hybridization groups. The probe bound to all strains tested except for the four strains of *A. salmonicida* (Table 4).

DISCUSSION

Our previous work has demonstrated that some strains of *Aeromonas* species possessed at least two type IV pilus structures, the Tap and bundle-forming pilus (Bfp) (3). The published genome sequences of *A. hydrophila* and *A. salmonicida* confirmed this by showing the genes for three distinct type IV pilus systems, Tap, Msh (Bfp), and a third as-yet-uncharacterized system, Flp, present on the chromosome (3, 26). Previous studies have suggested that the bundle-forming pilus of *A. veronii* bv. Sobria is a major adherence factor for diarrheagenic strains (14). However, until now genetic studies on this system in *Aeromonas* species have not been undertaken. Here, we have isolated the gene cluster encoding the complete *A. veronii* bv. Sobria bundle-forming type IV pilus system. Homology searches demonstrated that the system is similar to the mannose-sensitive hemagglutinin (MSHA) of *Vibrio cholerae* El Tor (17), and hence the *Aeromonas* genes were also designated *msh*. The N-terminal amino acid sequence of the protein encoded by the aeromonad *mshA* gene matched that of the previously reported major pilin sequence for both *A. veronii* bv. Sobria BC88 and the N-terminal sequences of other pili isolated from *Aeromonas* strains, placing it in the classical type A type IV pilus group (13). The gene organization of the *A. veronii* bv. Sobria *msh* locus matched that previously reported for *V. cholerae* (17), with the exception that there are two *mshI* genes in the *Aeromonas* chromosome, *mshI* and *mshI1*, that encode proteins equivalent to the N-terminal and C-terminal domains of the *V. cholerae* MshI protein, respectively. Database searches show that this arrangement can also be found in *A. hydrophila* and *A. salmonicida* as well as *Shewanella* species, whereas the single MshI arrangement is prevalent in most *Vibrio* and *Photobacterium* species.

The major pilin gene *mshA* was found in a gene cluster along with three other minor pilin genes, *mshB*, *mshC*, and *mshD*, and RT-PCR and *lacZ* fusions showed that these were transcribed in a single transcriptional unit from a promoter located upstream of *mshB*. The second promoter of the *msh* gene cluster was shown to be upstream of *mshI*, and this promoter gave a higher level of activity than the *mshB* promoter, which is similar to the observations in *V. cholerae* with regard to activity and arrangement (17). As the major and minor pilins are produced from the same mRNA, this would suggest that rate of translational initiation must vary between the separate *msh* pilin genes to give rise to the different amounts of pilin proteins. As in previous studies, we have detected only the major pilin protein (MshA) from purified pilin filaments and not the other minor pilin proteins (13).

The major pilin gene was mutated, resulting in a 10-fold or more decrease in adherence to both HEp-2 cells and biofilm formation to borosilicate glass (at 12 h). Furthermore, other mutations in the minor pilin proteins also had a great effect on biotic and abiotic adherence, suggesting that all the pilin proteins are required for a functional pilus structure for optimal adherence and that the pilins are not able to compensate for the loss of one of the others. Mutation of the *mshA* or *mshB* gene resulted in the absence of MshA or MshB protein on Western blots, further supporting the argument that both the major and minor pilin proteins are required for adherence.

The marked effect seen by mutating the bundle-forming pilus genes in *A. veronii* bv. Sobria BC88 is supported by our previous work that demonstrated that using the purified pili could block adhesion to both HEp-2 and Henle 407 cells and that anti-Bfp (MshA) antibodies also blocked adhesion to cell lines (14). Mutation of *tapD* had a dramatic effect on adhesion that was equivalent to the reduction observed for the mutations of the pilin structural genes. Furthermore, in the TapD mutant, we were unable to detect the presence of either MshA or MshB on Western blots, demonstrating the importance of this protein in MSHA biogenesis. The published aeromonad genomes to date all show one copy of the prepilin peptidase required for both maturation of the type IV pilin proteins and the pseudopilins required for the type II secretion system (T2SS). Therefore, as expected, mutation of *tapD* had a pleiotropic effect by inhibiting the secretion of various enzymes through the T2SS and by effecting aeromonad adherence and biofilm formation. The latter observations are possibly due to effects on the maturation of the Msh pilins or the Flp or Tap pilins.

The *mshH* gene has been annotated as part of the MSHA pilus system for a number of bacterial species, including *Photobacterium*, *Shewanella*, and *Vibrio* spp. Here, we have shown that the gene is not transcriptionally linked to the other pilin genes. The

gene encodes a protein that has GGDEF and EAL domains that are involved in di-guanylate cyclase synthase and phosphodiesterase activity, which are involved in making and breaking down c-di-GMP, respectively. The intracellular concentration of c-di-GMP modulates bacterial lifestyle choices from motile to sessile or vice versa and is important for adhesion and the formation of biofilms (7). Even though we see a slight decrease in the *A. veronii* bv. Sobria *mshH* mutant adhesion to HEp-2 cells, this is not supported by an inhibition in biofilm formation on borosilicate glass (Fig. 6), and this reduction is not like the greater than 80 to 90% drop in adhesion and biofilm formation that we observed for the other aeromonad MSHA mutants. Therefore, the *mshH* mutation is most likely having a more pleiotropic affect through the modulation of c-di-GMP concentrations, as reductions in c-di-GMP in another strain of *A. veronii* bv. Sobria have been shown to reduce adhesion and biofilm formation (23). This modulation could be affecting many other aeromonad factors associated with adhesion, including flagellar motility and outer membrane proteins (12, 22). Furthermore, the c-di-GMP network appears to be complex in the aeromonads, as the genome sequence of *A. hydrophila* demonstrated the presence of 13 proteins with GGDEF/EAL domains, 31 with only a GGDEF domain, and 9 with only an EAL domain (23, 26). Therefore, the mutation of MshH could possibly be compensated for by one of the many other GGDEF or EAL domain proteins.

Moreover, a homologue of *mshH* is found in enteric bacteria, such as *Serratia*, *Enterobacter*, and *Yersinia*, that do not possess an MSHA pilus system. Furthermore, the gene directly downstream of *mshH* in these bacteria is *mreB*, which is found downstream of the *msh* locus in *Aeromonas*, *Photobacterium*, *Shewanella*, and *Vibrio* spp. This suggests that the MSHA locus has been lost or deleted from this site in the enteric bacteria or that an ancestor of the closely related bacteria that possess this pilus system inserted it between *mshH* and *mreB*. A similar observation was also made by Marsh and Taylor, who suggested that the MSHA in *Vibrio cholerae* had been inserted into this particular locus due to the presence of a 7-bp direct repeat that flanks the locus (17). However, this evidence could further support the hypothesis that *mshH* is not a gene directly required for function of this type IV pilus.

Interestingly, the gene probe that encompassed part of *mshB* and all of *mshA* hybridized with all aeromonad mesophilic strains but no psychrophilic strains (*A. salmonicida*) tested, suggesting that the *msh* locus is present in all mesophilic strains but not in psychrophilic strains. This supports the evidence presented by Boyd et al. (3), who demonstrated that in *A. salmonicida* A449, there is a deletion in the *msh* locus between *mshN* and *mshD*, thus the area that the probe would have hybridized to has been deleted; this appears to be the case in other *A. salmonicida* strains also. The probe was able to demonstrate that the *msh* locus is present in mesophilic strains, whereas previously we were unable to do this, as the anti-Bfp antibody did not cross-react with 104 other strains of *Aeromonas* when tested (13), even though all previously isolated aeromonad long/wavy pili had very similar N-terminal amino acid sequences of the classical type A, type IV pilus group (13).

In conclusion, here we have undertaken the first genetic characterization of the mesophilic aeromonad bundle-forming pilus system, and through the use of defined mutants we have demonstrated the importance of this structure for adherence to tissue culture cells and biofilm formation. This supports our earlier findings that this structure is an important factor in aeromonad adherence.

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