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Physical Linkage of the *Vibrio cholerae* Mannose-Sensitive Hemagglutinin Secretory and Structural Subunit Gene Loci: Identification of the *mshG* Coding Sequence

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***Vibrio cholerae* O1 expresses a variety of cell surface factors which mediate bacterial adherence and colonization at the intestinal epithelium. The mannose-sensitive hemagglutinin (MSHA), a type IV pilus, is a potential attachment factor of the *V. cholerae* El Tor biotype. We describe a Tn*phoA* mutant that is defective in its ability to hemagglutinate mouse erythrocytes. The Tn*phoA* insertion maps to a recently identified genetic locus that encodes products that are predicted to be essential for assembly and export of the MSHA pilus. Insertional disruption at this locus in a *mshA-phoA* reporter strain provides evidence for a role of this locus in the latter stages of pilus assembly and/or export. These constructs have provided physical markers by which we have established close physical linkage of this secretion locus to a set of genes that includes the *mshA* structural gene. Sequence analysis of the intervening region between these two loci has revealed the presence of an open reading frame with homology to pilus biogenesis genes of several gram-negative bacteria. This genetic organization suggests an entire operon encoding the MSHA pilus and the components necessary for its assembly and secretion to the bacterial cell surface. The nomenclature of the MSHA structural and secretory locus has been redefined accordingly.**

Vibrio cholerae O1 is the etiologic agent of the acute diarrheal disease cholera. Pathogenic strains of *V. cholerae* O1 are divided into two biotypes, classical and El Tor, on the basis of a variety of phenotypic characteristics. Presently, the El Tor biotype is of greatest social and scientific significance because it is the prevalent biotype of the most recent pandemic. One feature which distinguishes the El Tor from the classical biotype is the expression of a mannose-sensitive hemagglutinin (MSHA) on the bacterial cell surface.

For *V. cholerae* to cause disease, the organism must attach to the intestinal epithelium, where it can colonize and secrete its potent exotoxin which is ultimately responsible for the massive fluid efflux characteristic of cholera illness. The toxin-coregulated pilus has been clearly established as a major determinant of classical biotype colonization (11, 28). The role of the toxin coregulated pilus in El Tor colonization has been controversial (12, 19, 22, 26), and moreover, colonization is likely to be a multifactorial process whereby several virulence determinants act coordinately to establish favorable interactions at the intestinal epithelium (14). Therefore, recent investigations have also focused on the role of MSHA in *V. cholerae* El Tor pathogenesis.

The gene encoding the structural subunit of MSHA was recently cloned and sequenced and found to reside within a cluster of genes that encode type IV pilin-like proteins (13). Recent work has demonstrated that proteins with extensive sequence similarity to type IV pili are involved in extracellular secretion and DNA uptake in a number of bacteria (25). It is therefore possible that the MSHA gene locus is involved in a number of bacterial functions, including attachment, colonization, and extracellular secretion. A gene locus proposed to be involved in MSHA secretion was also recently identified (10). This locus consists of eight open reading frames (ORFs), whose predicted gene products are homologs of several proteins known to be involved in the extracellular secretion of pullulanase by the bacterium *Klebsiella oxytoca* (21). Genetic disruption in any one of six of the eight ORFs was shown to abolish mannose-sensitive hemagglutination but did not interfere with the secretion of other products such as cholera toxin or extracellular protease (10).

In this study, we describe a Tn*phoA* mutant that is defective in its ability to hemagglutinate. The Tn*phoA* insertion maps to the fourth ORF of the MSHA secretory locus identified by Hase et al. (10). This insertion plus several additional genetic constructs was utilized to determine what role this ORF plays in the generation of the MSHA phenotype. Additionally, the Tn*phoA* insertion was used as a physical marker to investigate the location of the secretory genes with respect to the *mshA* structural gene. The results of this analysis revealed that these two sets of genes actually define a single locus. We propose that this genetic arrangement defines the MSHA biogenesis operon which is necessary for the expression, assembly, and extracellular secretion of the MSHA pilus.

MATERIALS AND METHODS

Bacterial strains and plasmids. All *V. cholerae* strains described in this study are derivatives of a spontaneous streptomycin-resistant mutant of a clinical isolate of the El Tor strain C6706 (Table 1). The MSHA-negative mutant DS577 was obtained by screening a collection of C6706 Tn*phoA* mutants for loss of the ability to hemagglutinate mouse CD-1 erythrocytes. Tn*phoA* mutagenesis was performed with the pRT291 delivery vector as described previously (27).

Mutant strain JM34 was generated by genetic backcross of the cloned Tn*phoA* insertion from DS577 into wild-type C6706 by use of the IncP1 allelic exchange method of Ruvkun and Ausubel (23). For this construction, *Escherichia coli* SM10 (24) carrying plasmid pJM61 (Table 1) was mated with C6706 and then selected on agar containing streptomycin and tetracycline. To select for chromosomal recombinants that had lost plasmid pJM61 but had retained the insertion due to homologous recombination into the chromosome, transconjugants were mated with strain MM294 carrying the IncPI plasmid pPH1JI (23) and then selected on agar containing streptomycin, kanamycin, and gentamicin.

The wild-type sequence corresponding to the region identified by the Tn*phoA* insertion was cloned by use of a linked marker strategy (20a). Initial sequence

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description[a]	Source or reference
Strains		
E. coli		
SM10	*thi thr leu tonA lacY supE recA* [RP4-2-Tc::Mu]	24
SM10λ*pir*	SM10λ*pirR6K*	27
MM294	*endA hsdR pro supF*	23
V. cholerae[b]		
C6706	Sm^r; wild-type El Tor, Inaba	Lab collection
DS577	Km^r; Tn*phoA* mutant; MSHA negative	This study
JM34	Km^r; Tn*phoA* backcross mutant	This study
JM2	Ap^r; pGP704 disruption mutant of ORF4	This study
JM4	Ap^r; pGP704 disruption mutant of ORF5	This study
JM53	*mshA-phoA* alkaline phosphatase gene fusion	This study
JM73	Ap^r; pGP704 disruption mutant of ORF4 in JM53	This study
Plasmids		
pJM61	Tc^r Km^r; pLAFR2 derivative containing 20-kb DS577 Tn*phoA* *Xba*I fragment	This study
pLAFR2	IncP1 Tc^r	8
pPHO7	$Ap^{r\prime}$ *phoA*	9
pPH1JI	Gm^r Sm^r *tra* IncP1	23
pGP704	Ap^r, suicide vector, *oriR6K mobRP4*	17
pCVD442	Ap^r *sacB*, pGP704 derivative	6
pJM62	pCVD442::*mshA-phoA*	This study

[a] Abbreviations: Tc^r, tetracycline resistance; Km^r, kanamycin resistance; Ap^r, ampicillin resistance; Sm^r, streptomycin resistance; Gm^r, gentamicin resistance.
[b] All *V. cholerae* strains are derivatives of C6706 *str1*, a spontaneous Sm^r mutant.

analysis was carried out with the United States Biochemical (Cleveland, Ohio) Sequenase kit. This sequence was utilized to construct genetic disruptions of ORF4 or ORF5 within the secretory locus by insertion of suicide vector pGP704 into the appropriate gene, similar to the procedure described by Miller and Mekalanos (17). In this procedure, an internal fragment of either ORF4 or ORF5 was inserted into pGP704, and the construct was recombined into the host chromosome, resulting in two incomplete copies of the target gene. The internal fragments were generated with PCR primers that flanked either an internal 220-bp fragment of ORF4 or an internal 170-bp fragment of ORF5. PCR primers carrying appropriate restriction enzyme sites were synthesized with a Milligen/Biosearch Cyclone Plus DNA synthesizer (model 8400; Perceptive Biosystems, Framingham, Mass.). Primer sequences for the ORF4 construct are as follows: 5′ sequence, 5′-CTAGTCTAGAGTCACCTACAGTTATGCAGG-3′; 3′ sequence, 5′-CTAGAATTTCGCCACTCTTTACCTGCCC-3′. Primer sequences for the ORF5 construct are as follows: 5′ sequence, 5′-CTAGTCTAGACATCG TGATCCTGTTGAAGC-3′; 3′ sequence, 5′-CTAGGAATTCGCCTTGGCTT CGACATCTC-3′. C6706 genomic DNA was utilized as template in a PCR containing 0.2 μM each primer per construct. The resulting products were digested with appropriate restriction enzymes and cloned into the multiple cloning site of the suicide vector pGP704 which is ampicillin resistant. SM10λ*pir* carrying each plasmid construct was mated with C6706, and exconjugants were selected on agar containing ampicillin and streptomycin. Homologous recombination by a single crossover event at either ORF4 or ORF5 in the *V. cholerae* chromosome yields a duplication of the internal ORF sequences carried on the plasmid, resulting in two incomplete, defective gene copies flanking the inserted pGP704 DNA. Mutants JM2 and JM4 were isolated in this manner to carry disruptions of ORF4 and ORF5, respectively.

A chromosomal *mshA-phoA* fusion was constructed in the following manner. Two sets of PCR primers were designed to amplify flanking regions of the *mshA* structural subunit gene. PCR products generated from these primers were cloned in frame to the *E. coli* alkaline phosphatase gene from plasmid pPHO7 (9) and then inserted into the pCVD442 (6) sucrase vector generating plasmid pJM62. The PCR-generated flanking regions allow homologous recombination of the *E. coli* alkaline phosphatase gene at the *mshA* gene locus in the *V. cholerae* chromosome. The following primer set, designated primer set 1, amplified an ~350-bp fragment that overlaps the 3′ end of *mshB* and the 5′ end of *mshA*: 5′ sequence, 5′-AAAAGTCGACAGCGAAAGCGAATAGTGG-3′; 3′ sequence, 5′-AAAAGGATCCATTGCACCAGCAACTGCACC-3′. The following primer set, designated primer set 2, amplified an ~250-bp fragment that overlaps the 3′ end of *mshA* and the 5′ end of *mshC*: 5′ sequence, 5′-AAAACTGCAGTCTA GATCCTGCAACGGTTGCTATGC-3′; 3′ sequence, 5′-AAAAGCATGCGT GGTTACCACCGCAAAGG-3′. Allelic exchange based on the *sacB* suicide plasmid pCVD442 was utilized to replace the chromosomal *mshA* gene with the alkaline phosphatase gene fusion in the following manner. SM10λ*pir* carrying pJM62 was mated with *V. cholerae* C6706. Progeny that had undergone genetic recombination at the *mshA* locus was selected on agar containing ampicillin and streptomycin as well as the chromogen XP (5-bromo-4-chloro-3-indoylphosphate). Blue, ampicillin-resistant exconjugants were plated on Luria-Bertani (LB) agar with 6% sucrose and no salt (13) to select for progeny that had undergone a second recombination event resulting in the loss of the pCVD442 vector sequences. A blue, sucrose-resistant colony that was ampicillin sensitive was selected and designated strain JM53. This *mshA-phoA* strain was then subjected to targeted gene disruption at ORF4 as described above, resulting in the construction of strain JM73.

Hemagglutination assay. Hemagglutination assays were carried out in 96-well round-bottom microtiter plates in a final volume of 0.2 ml. Bacteria (~10^7) were serially diluted in Krebs-Ringer buffer (7). CD-1 mouse (Charles River Laboratories) erythrocytes were collected in the presence of heparin, washed three times in Krebs-Ringer buffer, and resuspended at a final concentration of 1% in Krebs-Ringer buffer. An equal volume of bacteria was mixed with the mouse erythrocytes and incubated at room temperature for 1 h. Hemagglutination titers are expressed as the reciprocal of the highest bacterial dilution that gave strong hemagglutination (see Results).

Alkaline phosphatase assay. Alkaline phosphatase assays were performed on *mshA-phoA* fusions, strains JM53 and JM73, as described previously (4). Briefly, 0.05 ml of bacteria from an overnight culture was lysed in 1 ml of 1 M Tris-HCl (pH 8.0) containing 0.05 ml of 0.1% sodium dodecyl sulfate (SDS) and 0.05 ml of chloroform and then incubated at 37°C for 5 min. One-tenth milliliter of 0.4% *p*-nitrophenyl phosphate was added to each sample, and incubation was continued at 37°C until a yellow color appeared, at which time the reactions were stopped by the addition of 0.1 ml of 1 M KH_2PO_4. Incubation times were noted, and the optical densities at 420 and 550 nm (OD_{420} and OD_{550}) of the reaction mixture and the OD_{600} of the bacterial culture were read. Units of alkaline phosphatase activity are expressed by the following formula:

$$\text{alkaline phosphatase activity} = 1{,}000 \times \frac{OD_{420} - (1.75 \times OD_{550})}{\text{time (min)} \times \text{vol (ml)} \times OD_{600}}$$

Southern blot analysis. Verification of mutant constructs was performed by Southern blot analysis. Two probes were amplified by PCR for Southern blot analysis. Probe A (Fig. 1A), which spans ORF4 and the 5′ end of ORF5, was generated with the 5′ primer from the ORF4 genetic disruption protocol and the 3′ primer 5′-CTAGGAATTCGCCTTGGCTTCGACATCCTC-3′. Probe B (Fig. 1A), which spans the entire *mshA* coding region, was generated with the distal primers for the *mshA-phoA* gene fusion construction. The resultant products were random prime labeled with digoxigenin dUTP by use of the Boehringer Mannheim (Indianapolis, Ind.) Genius System Kit. Genomic DNA was isolated by phenol extraction (16) and digested with *Sma*I (unless otherwise noted) at 25°C for 3 h. DNA digests were run on 0.7% agarose gels and immobilized on Illuminator nylon membrane (Stratagene, La Jolla, Calif.) by capillary action and UV cross-linkage (Stratalinker; Stratagene). Hybridization was performed at 42°C overnight; high-stringency washes in 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at 65°C followed. Detection was carried out with alkaline phosphatase-conjugated antidigoxigenin antibodies and Lumiphos substrate (Boehringer Mannheim), followed by overnight exposure of XAR film (Eastman Kodak Co., Rochester, N.Y.).

Automated sequencing of intervening sequence. The PCR primers MSH6 and MSH7 were designed to flank the intervening region between *mshE* of the

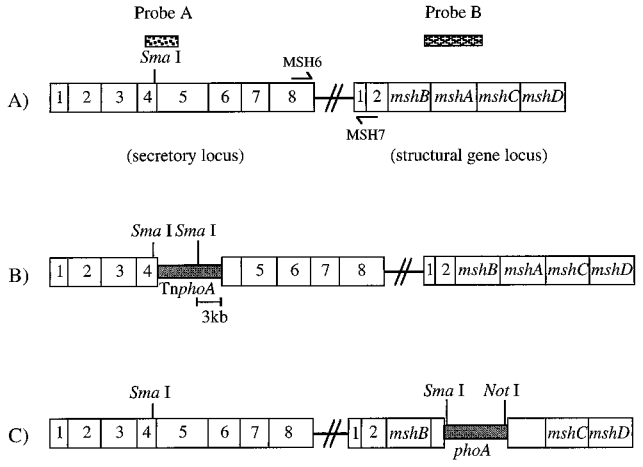

FIG. 1. Schematic representation of the MSHA secretory and structural gene loci. Open boxes indicate the locations and relative sizes of the ORFs. (A) Probes A and B utilized in Southern blot analysis span the stippled regions as indicated. A unique *Sma*I site is located within ORF4 of the secretory locus. The 1.3-kb intervening region is flanked by PCR primers MSH6 and MSH7, which were utilized to amplify this region for automated sequence analysis. (B) The DS577 Tn*phoA* insertion is located within ORF4 of the secretory locus. A *Sma*I site is located 3 kb upstream from the end of the Tn*phoA* insert. (C) Reporter construct *mshA-phoA* carries an additional *Sma*I site at the *mshA-phoA* junction and a unique *Not*I site near the end of the *phoA* insertion.

secretion locus and ORF1 of the *mshA* structural gene locus (Fig. 1A). The MSH6 primer sequence is 5′-AAAAGGATCCTTGCGGACAAATGATGCT-3′. The MSH7 primer sequence is 5′-AAAAGGATCCATGCCGCGACAAAC AC-3′. The 1.3-kb PCR product was gel purified (GeneClean; Bio 101, Inc., Vista, Calif.) and used as a template for automated DNA sequencing with the PRISM Ready Reaction Dye-Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, Calif.) primed with the flanking oligonucleotide PCR primers described above. The resulting sequence was utilized to generate two additional sets of internal oligonucleotide PCR primers to complete both strands of the entire 1.3-kb intervening region. All sequence analysis was performed with a model 373A automated DNA sequencer (Applied Biosystems).

Nucleotide sequence accession number. The DNA sequence generated has been entered in the GenBank database under the accession number U35009.

RESULTS

Isolation and characterization of MSHA mutants. The MSHA-negative mutant DS577 was identified by screening a collection of C6706 isolates carrying random Tn*phoA* insertions by a hemagglutination assay. The hemagglutination titer for the DS577 mutant was repeatedly 100-fold less than that of the wild-type strain. (Hemagglutination titers for strains C6706, DS577, JM34, JM2, and JM4 were 128, <1, 2, 8, and 8, respectively [results are means of three separate assays].) Sequence analysis demonstrated that the DS577 transposon insertion lies within ORF4 of a genetic locus previously proposed to encode products involved in the secretion of the MSHA pilus (10), which will be referred to as the MSHA secretory locus (Fig. 1). The sequence results also revealed that the *phoA* gene of the insertion was out of frame with ORF4, accounting for the extremely low level (4 U) of alkaline phosphatase activity produced by this strain (data not shown). Genetic backcross of the cloned Tn*phoA* insertion (see Materials and Methods) confirmed that the original MSHA-negative phenotype was due to the transposon insertion and not a secondary mutation. The backcross mutant JM34 displayed a 50- to 60-fold decrease in its hemagglutination titer (see data above). Since the Tn*phoA* insertion lies towards the 3′ end of ORF4, we were interested in investigating the ORF5 contribution to this phenotype. We therefore performed targeted gene disruption at ORF4 or ORF5 with the suicide vector pGP704 as described in Materials and Methods (17). The resulting mutants, JM2 and JM4, also exhibited a marked decrease in their ability to hemagglutinate, although at a consistently higher level than that observed in the original DS577 Tn*phoA* mutant or the backcross mutant JM34 (see above). Thus, disruption at either ORF4 or ORF5 of the secretory locus resulted in a MSHA-negative phenotype. These data are consistent with the findings of Hase et al. in which null alleles of several other ORFs within the proposed secretory locus also abolished mannose-sensitive hemagglutination (10).

Construction of a chromosomal *mshA-phoA* gene fusion. To determine the level at which the ORF4 gene product affects the MSHA phenotype, we constructed a *mshA-phoA* gene fusion in the *V. cholerae* C6706 chromosome and performed targeted gene disruption at ORF4 by the suicide vector strategy described above. While wild-type C6706 showed little endogenous alkaline phosphatase activity (33 ± 19 U [mean ± standard deviation]), the *mshA-phoA* fusion strain JM53 produced a high level of alkaline phosphatase specific activity (1,294 ± 24 U). JM73, the *mshA-phoA* gene fusion carrying the pGP704 vector insertion at ORF4, showed only a slightly lower alkaline phosphatase activity (985 ± 50 U) compared with that of the *mshA-phoA* gene fusion alone. This reduction in specific activity is not consistent with ORF4 playing a major regulatory role in *mshA* expression. The high activity elaborated by the MshA-alkaline phosphatase hybrid molecule in both strains suggests efficient localization of at least the alkaline phosphatase portion of the hybrid molecule across the inner membrane even in the absence of the ORF4 product. Thus, the phenotype of the ORF4 disruption is consistent with a role for this gene product in the later stages of pilus biogenesis, subsequent to initial secretion across the inner membrane.

Southern blot analysis. To verify the construction of the *V. cholerae* mutants in this study, we performed Southern blot analysis. Genomic DNA from mutant strains was subjected to restriction endonuclease digestion with *Sma*I. The wild-type C6706 chromosome carries a *Sma*I site at the 3′ end of ORF4 (Fig. 1A). Genetic alterations at this site can be detected easily with probe A (Fig. 1A), which spans ORF4 and the 5′ end of ORF5. Hybridization of this probe to *Sma*I-digested C6706 genomic DNA revealed the presence of two bands, a 20-kb band and a 16-kb band (bands 1 and 2, respectively) (Fig. 2A, lane 1). Hybridization of probe A to *Sma*I-digested DS577 genomic DNA detected an increase in the size of band 2 corresponding to the insertion of Tn*phoA* at ORF4 based on the following rationale. Sequence analysis demonstrated that

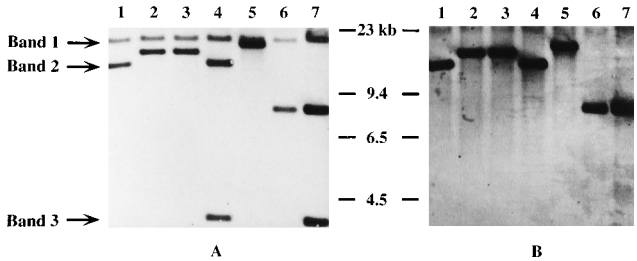

FIG. 2. Southern blot of *Sma*I genomic digests. (A) Hybridization with digoxigenin-labeled probe A. Lanes: 1, C6706; 2, DS577; 3, JM34; 4, JM2; 5, JM4; 6, JM53; 7, JM73. Band 1 is ~20 kb corresponding to the genetic fragment containing ORF4 of the secretory locus. Band 2 is ~16 kb corresponding to the genetic fragment containing ORF5 of the secretory locus. Band 3 is ~4 kb corresponding to the duplicated ORF4 sequences plus the inserted pGP704 vector. DNA size standards (λ*Hin*dIII; Gibco BRL, Bethesda, Md.) are indicated. (B) Hybridization with probe B of same blot as that shown in panel A.

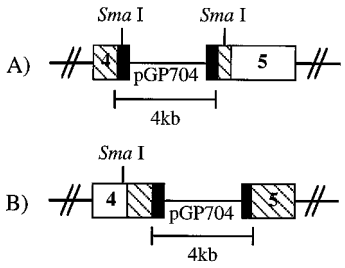

FIG. 3. Schematic representation of ORF4 (JM2) and ORF5 (JM4) genetic disruptions. (A) Insertion of pGP704 carrying an internal ORF4 fragment (▮) into the *V. cholerae* chromosome results in the production of two incomplete copies of the ORF4 gene and the duplication of the *Sma*I site. (B) Insertion of pGP704 carrying an internal ORF5 fragment (▮) into the *V. cholerae* chromosome results in the production of two incomplete copies of the ORF5 gene.

the Tn*phoA* insertion in DS577 lies just 3′ of the *Sma*I site at ORF4 (Fig. 1B). Insertion of Tn*phoA* at ORF4 would therefore result in an increase in size of the band (band 2) associated with ORF5. Because Tn*phoA* carries a *Sma*I site ~3.2 kb from its 3′ terminus, we would expect to see band 2 of DS577 increase in size by ~3.2 kb. This was observed for both the DS577 Tn*phoA* mutant and the backcross mutant JM34 (Fig. 2A, lanes 2 and 3). These data demonstrate that ORF5 resides on band 2 of the Southern blots hybridized with probe A.

The targeted gene disruptions at ORF4 and ORF5 were also confirmed by Southern blot analysis with probe A. Insertion of the pGP704 suicide vector into the *V. cholerae* chromosome at ORF4 results in the duplication of the internal targeted sequences of ORF4, including the *Sma*I site, as depicted in Fig. 3A. Southern blot analysis of genomic DNA isolated from this mutant, JM2, results in the production of three bands (Fig. 2A, lane 4). Band 1 and band 2 remain unchanged. However, insertion of pGP704 and the duplicated *Sma*I site results in an additional hybridizing band (band 3) (Fig. 2A, lane 4) corresponding to the pGP704 vector plus the duplicated ORF4 sequences. This finding is consistent with ORF4 residing on band 1 of Southern blots hybridized with probe A. Genetic disruption at ORF5 also results in the duplication of the targeted ORF5 sequences (Fig. 3B). In the case of the JM4 mutant, disruption of ORF5 results in a 4-kb increase in the size of band 2 which corresponds to insertion of the pGP704 vector plus duplicated ORF5 sequences (Fig. 2A, lane 5). In addition to confirming the construction of these mutants, these results demonstrate that band 1 lies 5′ of the *Sma*I site in ORF4, while band 2 lies 3′ of this site in Southern blots hybridized with probe A.

The *mshA-phoA* chromosomal insertion was confirmed by use of probe B (Fig. 1A). Insertion of *phoA* at the *mshA* structural gene results in the introduction of a second *Sma*I site (Fig. 1C). We therefore expected hybridization of probe B to *Sma*I-digested genomic DNA from the *mshA-phoA* fusion JM53 to result in the production of two bands. Southern blot analysis revealed a single band of 8 kb. We speculated that this 8-kb band was actually a doublet that could be resolved by performing a double digest with *Sma*I and *Not*I (Fig. 1C). A double digest with *Sma*I and *Not*I would release the 2.6-kb *phoA* fragment, resulting in a decrease in size of one of the 8-kb bands and the appearance of a 5.4-kb band. The Southern blot of *Sma*I- and *Not*I-digested genomic DNA from JM53 hybridized with probe B confirmed this prediction (Fig. 4A, lane 4).

Linkage analysis. Confirmation of the ORF4 disruption in the *mshA-phoA* genetic background suggested that the secretory locus was physically linked to the *mshA* structural subunit gene locus. This was apparent from the following results. When *Sma*I-digested genomic DNA from the *mshA-phoA* gene fusion JM53 was hybridized with probe A, band 2 was reduced in size by 8 kb (Fig. 2A, lane 6). Furthermore, JM73, the ORF4 disruption in the *mshA-phoA* genetic background, showed a similar banding pattern, with the addition of band 3 corresponding to the pGP704 vector and duplicated ORF4 sequences (Fig. 2A, lane 7). Since ORF5 lies within band 2 in *Sma*I genomic digests (see above) and insertion of *phoA* at the *mshA* structural gene results in the addition of a *Sma*I site (Fig. 1C), an alteration in the hybridization pattern of *Sma*I-digested genomic DNA from the *mshA-phoA* fusion JM53 would occur if the *mshA* gene locus was physically linked to the secretory locus. This hypothesis was confirmed by analysis of the *Sma*I genomic digests with probe B which hybridize to *mshA* sequences (Fig. 1A). Southern blot analysis with probe B of a *Sma*I genomic digest of C6706 revealed the presence of the 16-kb band corresponding to band 2 in the Southern blot with probe A (Fig. 2B, lane 1). The hybridization pattern of probe B consistently followed the pattern of band 2 observed for probe A in all the mutant backgrounds, confirming that the *mshA* structural gene is physically linked to the secretory locus.

Orientation of the MSHA secretory locus with respect to the *mshA* structural gene. To determine in what orientation the secretory locus lies with respect to the *mshA* structural gene locus, we compared the hybridization patterns from *Sma*I genomic digests and *Sma*I-*Not*I double digests of the *mshA-phoA* fusion JM53 with probe A (Fig. 4B). If the structural gene locus was arranged in an orientation opposite to that of the secretory gene locus, we would have expected band 2 to be reduced in size as a result of the excision of *phoA* sequences in a *Sma*I-*Not*I double digest (Fig. 1C). If, on the other hand, both loci were arranged in the same orientation, we would expect there to be no difference in the hybridization patterns observed for the *Sma*I versus the *Sma*I-*Not*I digests. Indeed, the banding pattern observed for the double digest hybridized with probe A was the same as that observed for the *Sma*I digest alone (Fig. 4B, lanes 2 and 4). This result establishes the directionality of the *mshA* secretory locus with regard to the *mshA* structural gene locus, i.e., the *mshA* secretory locus lies upstream of the *mshA* structural gene locus and the genes are all oriented in the same direction (Fig. 1A).

Intervening sequence analysis. PCR amplification of genomic DNA from C6706 with the primers MSH6 and MSH7 resulted in the production of a 1.3-kb product, consistent with the predicted distance between these regions by Southern analyses (data not shown). This product was utilized as template for sequencing with the MSH6 and MSH7 primers. Additional internal primers were generated as the sequence was extended

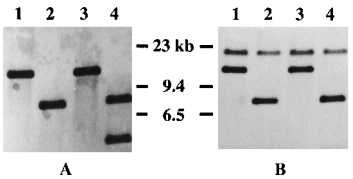

FIG. 4. Southern blots of the wild-type C6706 (lanes 1 and 3) and *mshA-phoA* fusion JM53 (lanes 2 and 4) strains. (A) Confirmation of *mshA-phoA* construct−hybridzation with probe B. (B) Orientation of the *mshA* structural locus−hybridization with probe A. Lanes: 1 and 2, *Sma*I genomic digests; 3 and 4, *Sma*I-*Not*I double digests. As described in the text, band 2 is detected with either probe as a single band migrating at 16 kb in the C6706 lanes or at 8 kb (as a single band detected with probe A or part of a doublet as detected with probe B) in the JM53 lanes. DNA size standards (λ*Hin*dIII; Gibco BRL) are indicated.

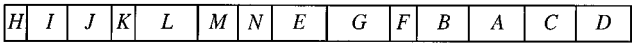

FIG. 5. Revised schematic of the MSHA genetic locus. The 12.2-kb locus is divided into 14 ORFs, all of which are oriented from left to right. Genes are designated initially with the nomenclature established by Jonson et al. (13) and then with genetic designations for ORFs described by Hase et al. (10) and those established in the present study.

to allow complete sequence analysis of both strands. Examination of the intervening region between the MSHA secretory and structural gene loci identified an ORF which we have termed *mshG* (Fig. 5). This gene is contiguous with the partial ORF1 sequence of the MshA structural gene locus reported by Jonson et al. (13). The *mshG* gene is 1,200 nucleotides in length and encodes a protein with a predicted molecular mass of 44,829 Da and an isoelectric point of 8.1. A database search of the MshG amino acid sequence by use of the BLAST program (2) revealed significant homology to the sequence of the pilus assembly gene of *Neisseria gonorrhoeae*, *pilG*, an integral cytoplasmic membrane protein (29). Topological analysis utilizing the TopPred II software for membrane protein structure predicts four membrane-spanning domains (5). In addition to the identification of *mshG*, we have completed the sequence of the *mshE* gene discovered by Hase et al. (10), extending this ORF by 120 nucleotides. The predicted protein product from this completed sequence exhibits identical homologies as reported previously; i.e., MshE is a putative nucleotide-binding protein with homology to PulE of the pullulanase secretory system (10).

DISCUSSION

We describe a *V. cholerae* El Tor-derived Tn*phoA* mutant, DS577, that is defective in its ability to hemagglutinate. The DS577 Tn*phoA* insertion maps to the fourth ORF of a potential MSHA secretory locus described by Hase et al. (10). In the Hase study, Tn*5* mutagenesis of ORF2, -3, -5, -6, -7, or -8 resulted in a MSHA-negative phenotype in each case. Our studies of DS577 and the ORF4 mutant derivatives JM2 and JM73 extend these findings. First, they further demonstrate the necessity of this locus in maintaining a functional MSHA pilus. Second, genetic disruption of ORF4 in the *mshA-phoA* reporter strain JM73 has no effect on alkaline phosphatase levels, thus providing experimental evidence to suggest that this locus is involved in later events of pilus expression, including assembly and secretion at the bacterial cell surface.

We have established that the *mshA* structural gene of the MSHA is physically linked to this secretory locus. Southern blot analysis with two probes of different specificities demonstrates that the MSHA structural gene locus and the secretory gene locus are separated by a 1.3-kb intervening region. Sequence analysis of this region revealed the presence of an ORF that is contiguous with the partial ORF1 sequence reported by Jonson et al. (13). In keeping with the previously established nomenclature, we have designated this ORF *mshG* and have identified the other unnamed ORFs accordingly (Fig. 5). Furthermore, we have changed the name of the secretory locus ORF5 from *mshD* to *mshL* to eliminate redundancy with the historically designated *mshD* of the structural locus.

The deduced MshG polypeptide has homology to several bacterial proteins essential for type IV pilus assembly and export, including PilG of *N. gonorrhoeae* (29) and PilC of *Pseudomonas aeruginosa* (18). While the exact role of these proteins in pilus assembly remains unclear, their predicted location within the cytoplasmic membrane and the proximity of their genes to those encoding either the structural subunit (*P. aeruginosa*) or the prepilin leader peptidase (*N. gonorrhoeae*) suggest a role in pilus assembly (18, 29). Unlike *mshG* of *V. cholerae*, the *pilG* gene of *N. gonorrhoeae* is located upstream of the gene encoding the prepilin peptidase PilD and is distal to the pilin subunit gene *pilE* (29). Recent investigations have demonstrated that type IV fimbrial biogenesis and function in the opportunistic pathogen *P. aeruginosa* are dependent upon a large number of genes which are located in three disparate regions of the bacterial chromosome and show homology to a network of proteins involved in the terminal stages of extracellular protein secretion (1, 15). Like *V. cholerae mshA*, the *P. aeruginosa pilA* structural subunit gene resides within a cluster of genes that encode proteins with consensus prepilin sequences; however, *pilA* is transcribed in the direction opposite to that of its ancillary genes (18). This transcriptional arrangement is different from what we describe for *mshA*. Our findings suggest that the genes responsible for the secretion and assembly of the MSHA pilus are all transcribed in the same orientation.

We propose that the MSHA structural and secretory loci together constitute a biogenesis operon which is necessary for the production, assembly, and secretion of functional MSHA pili. While no true promoters have been identified in this region, the organization of the 14 adjacent genes required for the production of the MSHA pilus suggests that expression of this locus could be driven by a single upstream promoter. The decreased hemagglutination titers of DS577 and JM34 compared with those observed for JM2 and JM4 are consistent with Tn*phoA* having a more-pronounced polar effect on downstream MSHA genes than that exhibited by the pGP704 insertion. This type of organization where the secretory-assembly components reside upstream of the pilin structural subunit gene has been reported for several *E. coli* fimbrial biogenesis operons (3, 20) but has never been observed for type IV pili. Further investigation of the putative MSHA biogenesis operon is necessary to elucidate its function with regard to *V. cholerae* El Tor pathogenesis.

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