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Nucleotide Sequence Encoding the Mannose-Fucose-Resistant Hemagglutinin of *Vibrio cholerae* O1 and Construction of a Mutant

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The region of DNA encoding the mannose-fucose-resistant hemagglutinin (MFRHA) of *Vibrio cholerae* O1 has been localized, and the nucleotide sequence has been determined. The region contains a single open reading frame encoding 230 amino acids, corresponding to a protein of 26.9 kDa. The N terminus of this protein is atypical for a protein localized in the outer membrane. A mutant lacking MFRHA activity has been constructed by allelic exchange after inactivation via the insertion of a kanamycin resistance gene cartridge. The MFRHA-negative mutant has been assessed for virulence in the infant mouse cholera model. This mutant shows a marked defect in its ability to persist in the infant mouse gut and is incapable of competing with the wild-type organism, even when given in 25-fold excess. This defect also leads to a >100-fold increase in the 50% lethal dose. These data suggest that the MFRHA is an important colonization factor in the infant mouse model.

In order to cause disease, *Vibrio cholerae* must first overcome the gastric barrier; next, it must colonize and adhere to the intestinal epithelium, and last, it must release its toxins in close proximity to their receptors. Pierce et al. (25) have shown that the colonizing capacity of *V. cholerae* is the major determinant of the immunogenicity of the organism.

Since colonization is an essential step in the process of infection, attention has recently turned to what factors may be responsible for adherence. Evidence so far has implicated a number of structures which could serve as adhesins. A number of hemagglutinins (HA) have been identified in *V. cholerae* and have been suggested as likely colonization factors. Finkelstein and Hanne (10) showed that all strains produced a soluble HA-protease. Apart from this soluble factor, there are a number of cell-associated components.

Several different cell-associated HA in *V. cholerae* have also been described (14, 15). These include the D-mannose-, D-fructose-sensitive HA expressed by El Tor strains, the L-fucose-sensitive HA expressed by classical strains, and the D-mannose-, L-fucose-resistant HA (MFRHA) expressed by both biotypes. Each HA differs from the others in numerous ways: spectrum of erythrocyte activity, sugar sensitivity pattern, calcium requirement, growth medium, and phase of expression.

We have previously isolated an *Escherichia coli* K-12 clone expressing the gene for an HA from *V. cholerae* classical Inaba 569B (13). The plasmid present in this clone was designated pPM471. By deletion analysis with both specific restriction endonucleases and *Bal* 31 nuclease, the gene was localized to a 0.72-kb region of DNA, implying a size of less than 27 kDa for the protein. Analysis of plasmids (in *E. coli* K-12 minicells) containing the cloned gene and deletion derivatives of these plasmids identified a protein of about 24 kDa, correlating with hemagglutinating activity. Using the cloned gene as a probe, we demonstrated the presence of homologous DNA in a variety of *V. cholerae* strains, including both biotypes. Furthermore, by screening gene banks in *E. coli* K-12 of *V. cholerae* El Tor O17, we isolated several El Tor clones containing this region of DNA and also expressing hemagglutinating activity.

This work describes the determination of the nucleotide sequence of the region encoding the MFRHA. An MFRHA mutant has been constructed, and its virulence has been assessed in the infant mouse cholera model.

MATERIALS AND METHODS

Bacterial strains and plasmids. *V. cholerae* 569B (classical Inaba), selected for motility, was obtained from laboratory stocks. *E. coli* K-12 strain LE392 (F^- *supF supE hsdR galK trpR metB lacY*) was obtained from L. Enquist. JM101 [F' (*traD36 proAB lacI*^{q} *lacZ* ΔM15) *supE* Δ(*lac-proAB*) *supF thi-1*] was obtained from J. B. Egan. *E. coli* K-12 strain S17-1 (*pro hsdR* RP4-2-Tc::Mu-Km::Tn*7*) was obtained from U. Priefer and employed for all conjugation experiments (9, 26, 30). Plasmid pBR322 (5) was used as the cloning vector. pRK290 (8) and pPH1JI (4) were obtained from J. Mekalanos. pPM1143 contains the Km^r cartridge from pUC4-K (38) cloned into pUC19 and was obtained from J. Hackett. Strains were grown in nutrient broth (Oxoid) for all experiments except bacteriophage propagation, for which 2 × YT broth was used (24), and in vitro competition assays, for which M9 minimal medium (23) was also used. All strains were maintained in 15% glycerol in 1% Bacto Peptone (Difco Laboratories, Detroit, Mich.) at −25 and −70°C for routine use and were lyophilized for long-term storage.

Hemagglutination assay. Cultures were shaken overnight at 37°C. Bacterial cells were sedimented by centrifugation at 5,000 × *g* for 10 min. The pellet was then suspended to a density of 10 A_{650} units/ml in modified Krebs-Ringer buffer (13). Bacteria were twofold serially diluted in round-bottomed microtiter plates (no. 1-221-24; Dynatech Laboratories, Inc., Alexandria, Va.) in 50 ml of modified Krebs-Ringer buffer. A 50-μl sample containing 1% washed

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erythrocytes was then added, the tray was tapped, and the erythrocytes were allowed to settle at 25°C for 1 h. The titer is defined as the reciprocal of the highest dilution in which hemagglutination was visible. All assays were performed in triplicate.

DNA isolation. Large-scale preparations of plasmid DNA were made by centrifugation of CsCl-ethidium bromide gradients of 0.5% Triton X-100-cleared lysates as previously described (8).

Small-scale preparations involved Triton X-100-cleared lysates of 10-ml cultures or 15-ml boiling preparations, both of which had been shaken overnight (for 18 h), as described by Holmes and Quigley (18). Chromosomal DNA was prepared as described elsewhere (23).

Transformation. Transformation of plasmid DNA into *E. coli* was performed with $CaCl_2$-treated cells as described by Brown et al. (7). Electroporation of plasmid DNA into *V. cholerae* was performed as described by Stoebner and Payne (32).

Enzymes. DNA polymerase I, alkaline phosphatase (molecular biology grade), and the restriction endonucleases *Bam*HI, *Bgl*II, *Cla*I, *Eco*RI, *Hin*dIII, *Mlu*I, *Nru*I, and *Xba*I were purchased from Boehringer Mannheim, Sydney, Australia. Nuclease *Bal* 31, T4 DNA ligase, *Eco*RI linkers, and *Cla*I linkers were purchased from New England BioLabs, Inc., Beverly, Mass.

Manipulations of DNA. Restriction endonuclease digestion and agarose gel electrophoresis were performed as described previously (13).

Southern DNA hybridizations. Chromosomal fragments hybridizing with the cloned DNA were detected by the procedure of Southern (31) as described elsewhere (22).

Bacterial conjugation. Exponential-phase cultures of the appropriate donor and recipient strains were mixed in a ratio of 1:10 and subsequently pelleted by centrifugation (4,500 × *g*, 5 min) and spread on cellulose acetate filters (Millipore Corp.) on a nutrient agar plate. After incubation for 4 to 5 h at 37°C, the cells were resuspended in 10 ml of saline (0.9% NaCl) and plated onto selective media.

Generation of fragments for nucleotide sequencing. The gene encoding the MFRHA has been localized within plasmid pPM1107, which still produces a hemagglutination-positive phenotype (13). Plasmid pPM1106 is an *Eco*RI-*Cla*I subclone of pPM471, and pPM1107 is pPM1106 with the *Mlu*I-*Mlu*I fragment deleted (13). Fragments for sequencing were generated by digestion with *Bal* 31 nuclease or *Taq*I and by using a series of synthetic oligodeoxyribonucleotides as primers with pPM1107 DNA. Use was made of the deletion plasmids which had been generated from both the *Eco*RI and *Cla*I ends by incubation in the presence of *Bal* 31 nuclease (13). The smallest plasmid (i.e., the largest deletion) still capable of mediating hemagglutination, pPM1127, was cleaved with *Cla*I and *Eco*RI and cloned into the *Acc*I-*Eco*RI sites of the replicative form of the M13 vector DNA (42). After transfection of JM101, white plaques were screened for inserts containing *V. cholerae* DNA. Plasmids pPM1128, pPM1129, and pPM1130 (these are derived from pPM1107 in the same manner as pPM1127 but display an HA-negative phenotype) (12) were also digested with *Cla*I and *Eco*RI and ligated to M13mp vectors digested with *Acc*I and *Eco*RI.

In addition, sequence information was obtained from *Taq*I fragments present in this region. The *Eco*RI-*Cla*I fragment of pPM1127 was extracted from a low-melting-point agarose gel. This isolated fragment was then incubated with *Taq*I at 65°C, and a sample of the digested DNA was end labelled with ^{32}P and Klenow fragment. This DNA was then added to the remaining digested sample and run on a 6% polyacrylamide gel. DNA bands were visualized by autoradiography after 30 min, and the appropriate bands were excised from the gel and used for cloning into M13mp vectors which had been cleaved with *Acc*I. *Taq*I digestion of the *Eco*RI-*Cla*I insert of pPM1127 generated four bands with sizes of 62, 168, 241, and 652 bp. In order to sequence the complementary strand in the 5′-to-3′ direction, synthetic oligonucleotides were made by using an Applied Biosystems model 381A DNA synthesizer. These oligonucleotides span nucleotides 645 to 627, 500 to 482, 410 to 392, and 150 to 132.

Sequencing reactions were carried out by the dideoxy chain termination procedures of Sanger et al. (27, 28). Randomly terminated chains were separated on ultrathin 6% polyacrylamide gels in the presence of 8 M urea.

Infant mouse virulence assay and colonization. The virulence of strains was determined by using the infant mouse model as described by Attridge and Rowley (2).

Colonization experiments were performed by orally administering, simultaneously and in different ratios, the two strains to be tested. Groups were of sufficient size that triplicate samples could be obtained for each time point. At given times, three mice were sacrificed, their entire intestines were removed, and *V. cholerae* organisms were enumerated. In vitro competition assays were performed by mixing exponential-phase cultures of the two strains in different ratios in nutrient broth or M9 minimal medium and incubating at 37°C. Samples were taken at 0, 3, and 6 h for counting of viable organisms in the presence and absence of kanamycin.

Nucleotide sequence accession number. The nucleotide sequence of the region encoding the MFRHA has been deposited in the EMBL, GenBank, and DDBJ data bases under accession number X64097.

RESULTS

Nucleotide sequence determination. The sequence of a 775-bp region encoding the MFRHA gene is shown in Fig. 1. An open reading frame extends throughout most of the DNA sequence, with potential initiation codons beginning at nucleotides 37, 76, and 94. The open reading frame corresponds to translated products of 26.9, 25.6, and 24.8 kDa. The size of the product observed in minicells is about 24 kDa (13).

Since the MFRHA is clearly a surface-associated protein (as bacteria expressing it mediate hemagglutination of erythrocytes), it is expected to have an NH_2-terminal signal sequence to facilitate its export out of the cytoplasm. However, a typical signal sequence is not apparent (39, 40). The overall character of the protein is basic, with a predicted pI ranging from 8.92 to 9.83 (depending on the initiation codon used), and is reminiscent of a cationic outer membrane protein, such as OmpH of *Salmonella typhimurium* (20), which effectively floats in the lipopolysaccharide layer and is anchored by charge interactions. Certainly, a hydropathy plot does not reveal any particularly marked hydrophobic domains (Fig. 2).

Construction of the MFRHA-negative mutant, V762. In order to analyze the function of the MFRHA gene product, a mutation eliminating this gene has been constructed in *V. cholerae*. The mutation in the MFRHA gene was constructed via the replacement of the 0.7-kb *Xba*I fragment of pPM471 (13) with a kanamycin resistance cartridge, creating plasmid pPM1146 (Fig. 3). This insertion abolishes MFRHA

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  1  TAA TGA CAG ATA CGA TTT TAA CGT ATC TGT CAT TTC ATG TTA GGT TAT    48
  1                                                Met Leu Gly Tyr     4

 49  GCG TTT CCC TCG ATA TTA GGC AAT TTA ATG TCA AAA ATT TAT CAA ATG    96
  5  Ala Phe Pro Ser Ile Leu Gly Asn Leu Met Ser Lys Ile Tyr Gln Met    20

 97  GAT GCG GTT GAT TGG CTT AAA ACA CTT GAA AAT TGT AGT GTT GAT CTG   144
 21  Asp Ala Val Asp Trp Leu Lys Thr Leu Glu Asn Cys Ser Val Asp Leu    36

145  TTC ATC ACT GAT CCA CCA TAT GAA TCG CTA GAA AAA TAT AGA CAA ATA   192
 37  Phe Ile Thr Asp Pro Pro Tyr Glu Ser Leu Glu Lys Tyr Arg Gln Ile    52

193  GTT ACG ACT ACA CGG TTA AAA GAG AGT AAA TCA TCG AGC AAT CAA TGG   240
 53  Val Thr Thr Thr Arg Leu Lys Glu Ser Lys Ser Ser Ser Asn Gln Trp    68

241  TTT AGT GTT TTT CCT AAC ACT AGG TTT GAA GAG TTG TTT CGT GAA GTT   288
 69  Phe Ser Val Phe Pro Asn Thr Arg Phe Glu Glu Leu Phe Arg Glu Val    84

289  TAT AGA GTG CTA AAA AAA GGT TCT CAT TTC TAT TTA TTT TGC GAC CAG   336
 85  Tyr Arg Val Leu Lys Lys Gly Ser His Phe Tyr Leu Phe Cys Asp Gln   100

337  GAA ACT ATG TTT TTG GCG AAA CCA ATA GCG GAA AGT GTA GGC TTT AAA   384
101  Glu Thr Met Phe Leu Ala Lys Pro Ile Ala Glu Ser Val Gly Phe Lys   116

385  TTT TGG AAG CCT ATA GTT TGG GAT AAG TGT CGT ATA GGT ATG GGA TAT   432
117  Phe Trp Lys Pro Ile Val Trp Asp Lys Cys Arg Ile Gly Met Gly Tyr   132

433  CAT TAT CGT GCT AGA TAT GAA TTT ATT CTA TTT TTC GAG AAA GGA AAG   480
133  His Tyr Arg Ala Arg Tyr Glu Phe Ile Leu Phe Phe Glu Lys Gly Lys   148

481  AGA AAG TTA AAT GAT TTA AGT GTT CCT GAT GTG TTG GAA TAT AAG AGA   528
149  Arg Lys Leu Asn Asp Leu Ser Val Pro Asp Val Leu Glu Tyr Lys Arg   164

529  GTT TGG AAA GGC TAC CCA ACA GAA AAG CCA GTT GAA CTT CTG GAG GTT   576
165  Val Trp Lys Gly Tyr Pro Thr Glu Lys Pro Val Glu Leu Leu Glu Val   180

577  TTG ATT AGG CAA AGC TCT TCA GAA AAT GAA ATT GTA GCG GAT TCA TTT   624
181  Leu Ile Arg Gln Ser Ser Ser Glu Asn Glu Ile Val Ala Asp Ser Phe   196

625  TTT GGT TCA GGC GCA ACT TTA ATT GCA GCT AAT AAT CTC TCT AGA AAA   672
197  Phe Gly Ser Gly Ala Thr Leu Ile Ala Ala Asn Asn Leu Ser Arg Lys   212

673  TAC ATT GGT TGT GAT ATA TCA ATG TCT GCA CAT GAG TAT TTT AAG AAT   720
213  Tyr Ile Gly Cys Asp Ile Ser Met Ser Ala His Glu Tyr Phe Lys Asn   228

721  AGA GCT TGA AAC TAT TTA TGC CTA ACA AAC GCC TCA AGA GGG ACT GTC   768
229  Arg Ala ***                                                     231

769  AAC GCG T                                                       775
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FIG. 1. Nucleotide sequence of the region encoding the MFRHA. The translated sequence of the open reading frame corresponding to the MFRHA gene is shown. The termination codon is designated with asterisks. A region of inverted repeat symmetry preceding the gene is underlined.

activity in *E. coli* K-12. When hemagglutination assays were performed as described in Materials and Methods with *E. coli* K-12 strain LE392 containing recombinant plasmids, plasmids pBR322, pPM471, pPM1127, and pPM1146 gave hemagglutination titers of 0, 16, 16, and 0, respectively. The insert of pPM1146 was subcloned into the mobilizable vector pRK290, giving plasmid pPM1147 (Fig. 3), to allow introduction of the mutation into the *V. cholerae* chromosome by homologous recombination (1, 11). The mutation was confirmed by Southern hybridization (31).

Properties of an MFRHA-negative mutant. Before the effects of the MFRHA mutation on virulence were examined, the mutant was compared with its parent strain for hemagglutinating activity (Table 1), motility, and ability to exhibit chemotaxis towards glucose and L-methionine. It was also examined for other properties which could affect virulence, such as auxotrophy, lipopolysaccharide structure, and cholera toxin synthesis. It was in all cases identical to its parent strain (data not shown).

Virulence in the infant mouse cholera model. In order to assess the effect of the MFRHA mutation on virulence, the 50% lethal doses (LD_{50}s) of 569B and its MFRHA-negative variant, V762, were determined. Whereas the parent strain has an LD_{50} of 9×10^5 at 48 h, the mutant is effectively attenuated, with an LD_{50} of $>1 \times 10^8$ (Table 1). Indeed, only mild diarrhea was observed with doses of up to 5×10^8 of the mutant, V762.

To determine that the attenuation described above is due to the MFRHA defect, an attempt was made to complement the defect by introducing the smallest clone retaining MFRHA activity in *E. coli* K-12, pPM1127 (13), into V762. Neither hemagglutinating activity nor virulence was restored (Table 1), the implications of which are discussed below.

In order to compare the ability of V762 to survive and colonize in vivo with that of its parent, 569B, groups of four infant mice were fed with doses corresponding to either about 0.1 or 10 LD_{50}s of the parent strain. At 20 h and 44 h, two mice from each group were sacrificed and the *V. cholerae* organisms in the entire intestines were enumerated (Fig. 4). At 44 h, the mice fed the high dose of the wild type, 569B, were moribund. Bacterial counts showed that 569B could persist and grow, whereas the numbers of V762 declined rapidly and appeared to stabilize at approximately 500-fold fewer organisms than the parent, 569B. This inability to compete is not apparent with competition experiments performed in vitro, in which ratios of 569B to V762 do not alter significantly over the course of the experiments (data not shown). Thus, V762 was not totally defective in its ability to colonize, but it was markedly affected.

One possible explanation for the inability of V762 organisms to reach the numbers that 569B can reach in the intestine is that V762 lacks an undefined factor which is necessary for growth and persistence, and it consequently is a poor colonizer. To assess whether this factor can be provided by cross-feeding, infant mice were fed the two strains in combination in different ratios. From the data in Table 2, it is clear that V762 is unable to compete with 569B for survival in the gut, even when inoculated at a 25-fold-higher dose.

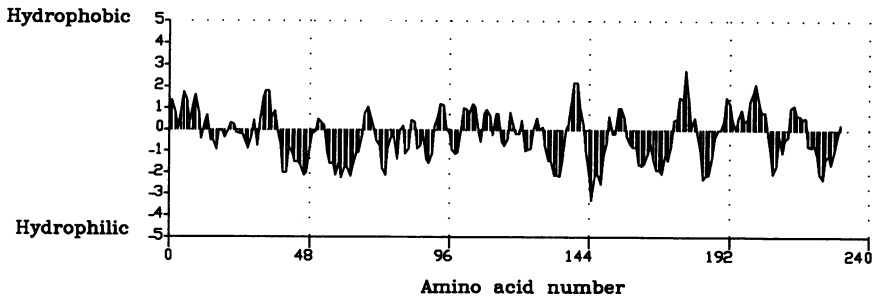

FIG. 2. Hydropathic nature of the MFRHA protein. The hydropathy plot of the MFRHA protein, based on the predicted amino acid sequence, was determined by the method of Kyte and Doolittle (21) by using a window of six amino acids.

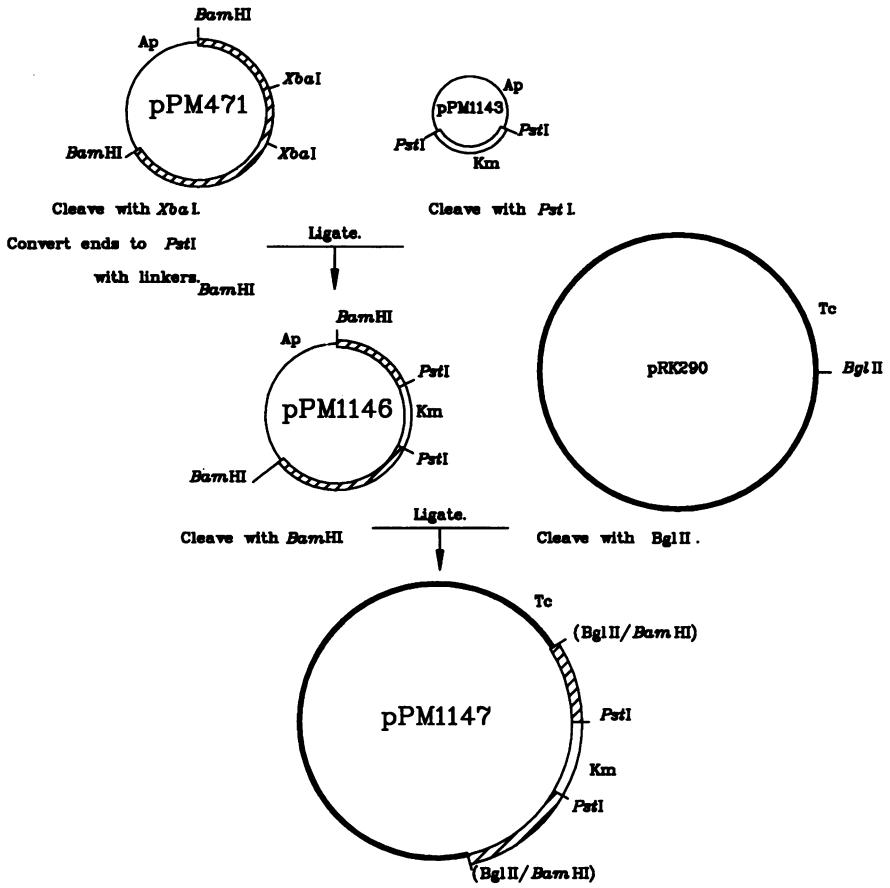

FIG. 3. Construction of a mutation in the MFRHA gene. Plasmid pPM471 was cleaved with *Xba*I, and the ends were filled by using the Klenow fragment of DNA polymerase I. Phosphorylated *Pst*I linkers were ligated onto the blunt ends. After partial digestion with *Pst*I, the DNA was ligated to the *Pst*I fragment of pPM1143 containing the kanamycin resistance gene. Transformants were selected on nutrient agar containing ampicillin and kanamycin to yield plasmid pPM1146. The entire *Bam*HI fragment of pPM1146 was then cloned into the broad-host-range vector pRK290, which had been cleaved with *Bgl*II, and then transformed into the *E. coli* K-12 streptomycin-resistant strain S17-1 (29) with selection for kanamycin and tetracycline resistance, to yield pPM1147. This plasmid can be mobilized at high frequency into various gram-negative organisms by using a helper plasmid. The resultant plasmid, pPM1147, was then transferred to *V. cholerae* 569B. The Gm^r plasmid pPH1JI (4) was transferred to strain 569B(pPM1147) by conjugation in order to cure the strain of pPM1147 by incompatibility. The pPH1JI plasmid was subsequently chased out by using a further incompatible plasmid, pME305 (25), which could be readily cured because of its temperature-sensitive replicon. The resultant strain was V762, which now contained the Km^r cartridge instead of the *Xba*I fragment. This was confirmed by Southern hybridization (data not shown).

DISCUSSION

V. cholerae is a noninvasive intestinal pathogen, and consequently, adherence and colonization are critical factors in the disease process. The toxin-coregulated pilus has been shown to be essential for this colonization (17, 29, 34). Data presented here suggest that the MFRHA may be an additional colonization factor, at least in the infant mouse model.

TABLE 1. Comparison of the virulence in infant mice of strain 569B and its MFRHA-negative mutant

Strain	Hemagglutination titer[a]	LD_{50}[b]
569B	16	9×10^5
V762	0	$>1 \times 10^8$
V762(pBR322)	0	$>1 \times 10^8$
V762(pPM1127)	2	$>1 \times 10^8$

[a] See Materials and Methods.
[b] Assessed at 48 h after oral administration of the bacteria.

The MFRHA was originally cloned in this laboratory by virtue of cross-reactivity with antiserum raised against the soluble HA-protease (13) by Finkelstein and Hanne (10). The possibility that a distinct HA had been cloned was raised, as the clone did not exhibit the protease activity characteristic of the HA-protease (12). The nucleotide sequence reported here confirms that the two proteins are distinct entities, as this sequence bears no similarity to that reported for the HA-protease (16). The MFRHA has also been cloned (35) and sequenced by van Dongen and coworkers (36). Six differences between the two sequences (not shown), all of which lead to amino acid substitutions, are observed. Three of these are conservative, and none of them significantly alter the character of the protein as reported here. However, van Dongen et al. (36) also assert that the MFRHA is not the 27-kDa protein whose sequence is reported here but a 7-kDa protein which lies immediately upstream (3, 36). In order to investigate their assertion, which is based on the hemagglutination titers of various constructs introduced into *E. coli* K-12 (36), we have repeated the experiments done with

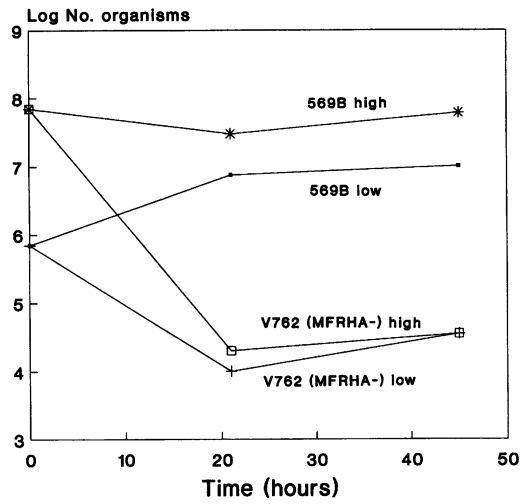

FIG. 4. Colonization of infant mice. Infant mice were fed 5×10^5 cells (low dose) or 4×10^7 cells (high dose) of either 569B or its MFRHA-negative mutant, V762. At 21 and 44 h, the mice were sacrificed, their entire intestines were removed, and the numbers of *V. cholerae* organisms were determined.

strain LE392 (see above) but with the host strain used by van Dongen and coworkers (HB101) (6), with identical results (data not shown). The resolution of this problem is the subject of current investigations.

The maturation and/or localization of the MFRHA is also intriguing. The MFRHA clearly must be located on the cell surface because of its ability to mediate hemagglutination, but it possesses an unusual NH_2 terminus. It is possible that an alternative signal peptidase is required for processing. One possibility is TcpJ, which is responsible for the maturation of TcpA (19). A similar signal peptidase has been predicted to be involved in processing a number of proteins in other organisms (41). However, there are also precedents for other surface proteins not having defined signal sequences for their export. For example, the Ipa proteins involved in adherence and invasion in *Shigella* spp. appear to be localized to the surface independent of an NH_2-terminal signal sequence (37). Indeed, these proteins are also markedly hydrophilic. In light of the discrepancy mentioned above, it should be noted that the 7-kDa protein also does not possess a typical signal sequence, nor does it show marked hydrophobicity (3, 36).

TABLE 2. Competition between 569B and its MFRHA-negative mutant

Expt no.	Strain	Dose[a]	Dose ratio[b]	Recovery[a,c]	Recovery ratio[b]
1	569B	2×10^5	4	2×10^5	1,333
	V762	5×10^4		1.5×10^2	
2	569B	2×10^5	0.4	1.6×10^6	533
	V762	5×10^5		3×10^3	
3	569B	2×10^4	0.04	0.95×10^5	238
	V762	5×10^5		4×10^3	

[a] Organisms per mouse.
[b] Number of 569B organisms/number of V762 (MFRHA-negative mutant) organisms. Total bacteria were enumerated on nutrient agar, and V762 counts were determined by replica plating onto nutrient agar containing kanamycin.
[c] Recovery was at 24 h after feeding the dose.

The decrease in virulence and the concomitant loss of MFRHA activity observed with 569B on introduction of the mutation described here suggest a role for the MFRHA in colonization. However, the inability to complement the MFRHA mutant in *V. cholerae* with the smallest cloned fragment active in *E. coli* K-12 suggests the possibility of an additional mutation. We have assayed a wide range of factors that could possibly cause this defect (see Results) and find none of them to be defective. The mutation introduced here also removes most of an open reading frame immediately downstream of the MFRHA gene (3), and it is possible that the loss of this gene is (at least) partially responsible for the phenotype or, alternatively, that the insertional inactivation has a polar effect on downstream genes (whose functions remain unknown). The construction of mutations that avoid this pitfall should help resolve this problem. The attenuation is unlikely to be due to the cost of kanamycin resistance, as insertional inactivation with the same kanamycin resistance cartridge in the extracellular DNase gene *dns* (11) and the lipopolysaccharide Ogawa specificity gene *rfbT* (33) does not affect virulence.

Taylor et al. (34) have previously shown that *tcpA* mutants unable to synthesize the toxin-coregulated pilus are defective in colonization in infant mice. Such mutants are also unable to effectively compete with the wild-type bacteria in vivo, whereas toxin-defective mutants are relatively unaffected. The MFRHA mutant was not totally lacking in its ability to colonize, and a basal level was maintained. Perhaps this is due to the toxin-coregulated pilus or a further colonization factor, and it would therefore be of interest to examine the effects of a double mutant defective in both the toxin-coregulated pilus and the MFRHA.

A detailed analysis of the MFRHA and its linked genes may facilitate a better understanding of the biogenesis of the MFRHA and its localization onto the cell surface as well as aid in defining its role in the course of a cholera infection. This is currently being undertaken.

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