Molecular Cloning and Expression in *Escherichia coli* K-12 of the Gene for a Hemagglutinin from *Vibrio cholerae*

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Using antiserum to the purified soluble hemagglutinin we isolated an *Escherichia coli* K-12 clone expressing the gene for a hemagglutinin from *Vibrio cholerae* 569B. The plasmid present in this clone was designated pPM471. By deletion analysis with both specific restriction endonucleases and *Bal* 31 nuclease, we localized the gene, to a 0.72-kilobase region of DNA, implying a molecular weight of less than 27,000 for the protein. Analysis in *E. coli* K-12 minicells of plasmids containing the cloned gene and deletion derivatives of these plasmids identified a protein of 24,000 daltons 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.

The disease cholera is caused by *Vibrio cholerae* of the O1 serotype, which can be further subdivided into two biotypes: classical and El Tor. The classical biotype was responsible for six pandemics up until 1961, when the onset of the seventh pandemic began and organisms of the El Tor biotype were found to be responsible (3, 20). In 1963 Finkelstein and Mukerjee (11) observed that vibrios of the El Tor biotype grown on solid medium could agglutinate chicken erythrocytes (RBCs), whereas strains of the classical biotype could not. This characteristic of El Tor strains in conjunction with resistance to polymyxin B (13, 30), sensitivity to Mukerjee's type IV phage (27), and the production of soluble hemolysin (20), were the four distinguishing features which allowed biotype differentiation.

Since 1961 when Bales and Lankford (Bacteriol. Proc. Abstr. M64, p. 118, 1961) suggested that the interaction between *V. cholerae* and RBCs mimics that of the organism with the intestinal epithelium, a number of workers have become interested in the various hemagglutinins of *V. cholerae* and their properties.

Hanne and Finkelstein (15) have described four distinct hemagglutinins. Strains of both biotypes possess at least one major cell-associated hemagglutinin (19). The El Tor cell-associated hemagglutinin is inhibited by D-mannose and D-fructose, and this is the hemagglutinin used in biotyping *V. cholerae*. The classical cell-associated hemagglutinin is inhibited by L-fucose. In addition to the cell-associated hemagglutinins, a soluble hemagglutinin has been detected in culture supernatants. This hemagglutinin is not inhibited by any sugars tested (15) and has been found in all strains regardless of their biotype. Each hemagglutinin differs with respect to the others in numerous ways: spectrum of RBC activity, sugar sensitivity pattern, Ca^{2+} requirement, and phase of expression.

Of the hemagglutinins, the soluble hemagglutinin is the one which has been studied in greatest detail. Finkelstein and Hanne (10) have purified the protein and shown that in addition to the ability to agglutinate RBCs this protein has a number of other biological activities. It has protease activity able to hydrolyze fibronectin and ovomucin and cleave lactoferrin (9). It also nicks and activates the A subunit of the

cholera enterotoxin (5). The purified protein has also been shown to inhibit attachment of *V. cholerae* to the intestinal epithelium (8, 10) and therefore may be one of the factors involved in adherence of *V. cholerae* to the intestinal epithelium.

Because of its apparent role in the pathogenesis of *V. cholerae*, we sought to clone the gene encoding the soluble hemagglutinin as a preliminary to constructing specific mutants for analyzing its role.

MATERIALS AND METHODS

Bacterial strains and plasmids. *V. cholerae* 569B (classical Inaba) and O17SR (El Tor Ogawa) were obtained from D. Rowley. *Escherichia coli* K-12 LE392 (F⁻ *supF supE hsdR galK trpR metB lacY*) was obtained from L. Enquist, *E. coli* and DS410 (F⁻ *minA minB rpsL*) was from D. Sherratt. Plasmid pBR322 (4) was used as the cloning vector. All strains were maintained in 15% glycerol in 1% Bacto-Peptone (Difco Laboratories, Detroit, Mich.) at −25 and −70°C for routine use and lyophilized for long-term storage.

Media and buffers. Brain heart infusion broth (Difco) was the standard medium for *V. cholerae* strains and nutrient broth (Difco) was used for the *E. coli* K-12 derivatives. Modified Krebs-Ringer buffer (12) was used routinely.

RBCs. RBCs were obtained from BALB/c mice. The blood samples were collected in 3.8% sodium citrate and washed three times in modified Krebs-Ringer buffer before use in hemagglutination experiments. Human RBCs of blood groups O, A, and B were also used.

Antiserum. Rabbit antiserum to the purified soluble hemagglutinin was generously provided by R. A. Finkelstein.

Absorption of antiserum. Rabbit antiserum against purified hemagglutinin was twice absorbed with 10 mg of cell envelope material per ml extracted from *E. coli* K-12 LE392 and twice absorbed with LE392 live bacteria (10^8/ml).

Hemagglutination assay. Cultures were shaken overnight at 37°C. Bacterial cells were sedimented by centrifugation at $5,000 \times g$ for 10 min. The pellet was then suspended in 1 ml of modified Krebs-Ringer buffer. Bacteria were twofold serially diluted in round-bottomed microtiter plates (no. 1-221-24; Dynatech Laboratories, Inc., Alexandria, Va.) in 50 μl of modified Krebs-Ringer buffer. A 50-μl sample of 1% washed RBCs was then added, the tray was tapped, and the

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RBCs were allowed to settle at room temperature for 1 h. The titer is defined as the reciprocal of the highest dilution in which hemagglutination was visible.

Hemagglutination inhibitions. To test for inhibition of hemagglutination, we used a suspension of bacteria adjusted to contain 2 hemagglutinin titer doses. Sugars (10 mg/ml in modified Krebs-Ringer buffer) were serially diluted in micro-titer plates. Hemagglutinin suspension was then added and allowed to interact for 30 min at room temperature. RBCs were then added and incubated for a further 60 min, after which the trays were read. Sugars tested include D-mannose, D-fructose, D-glucose, D-galactose, and L-fucose.

DNA isolation. Large-scale preparations of plasmid DNA were performed by centrifugation of CsCl-ethidium bromide gradients of 0.5% Triton X-100-cleared lysates as previously described (7, 25).

Small-scale preparations involved Triton X-100-cleared lysates of 10-ml shaken overnight cultures or boiling 15-ml shaken O/N (18-h) cultures as described by Holmes and Quigley (18). Chromosomal DNA was prepared by a method to be described elsewhere (P. A. Manning, M. W. Heuzenroeder, J. Yeadon, D. I. Leavesley, P. R. Reeves, and D. Rowley, submitted for publication).

Transformation. Transformation of plasmid DNA into *E. coli* was performed with CaCl$_2$-treated cells as described by Brown et al. (6).

Enzymes. DNA polymerase I, alkaline phosphatase (molecular biology grade), and restriction endonucleases BamHI, BglI, ClaI, EcoRI, HindIII, MluI, NruI, and XbaI were purchased from Boehringer Mannheim, Sydney, Australia. Nuclease Bal 31, T4 DNA ligase, EcoRI linkers, and ClaI linkers were purchased from New England BioLabs, Inc., Beverly, Mass.

Digestion of DNA. Digestion of DNA by restriction endonucleases was performed in a final volume of 20 μl which contained approximately 0.5 μg of DNA incubated with 1 U of each restriction enzyme at 37°C for 2 h. The enzyme was inactivated by heating for 10 min at 65°C. Tracker dye was then added (13% Ficoll, 1 mg of bromophenol blue per ml).

Agarose gel electrophoresis. DNA was run on an agarose gel (130 by 149 by 5 mm). All gels were run at room temperature submerged in TBE buffer (67 mM Tris base, 22 mM boric acid, 2 mM EDTA, pH 8.8) at 100 V for 4 to 5 h. Gels were then stained with 2 μg of ethidium bromide per ml in distilled water and photographed on Polaroid 667 positive film or 665 negative film with an Oliphant transilluminator UV light source.

Calculation of restriction fragment size. The sizes of restriction enzyme fragments could be calculated by comparison with *Bacillus subtilis* bacteriophage SPP1 DNA cut with EcoRI. The sizes of the standards used differ from those published (28) and have been calculated by us using bacteriophage lambda and plasmid pBR322 as standards, using the program DNAFRAG (29). The sizes (kilobases [kb]) used are: 8.37; 7.2; 6.05; 4.9; 3.55; 2.63; 1.73; 1.61; 1.29; 1.19; 0.99; 0.86; 0.63; 0.48; 0.38.

Construction of gene banks. The gene banks of *V. cholerae* strains constructed in pBR322 have been previously described (25). The gene banks constructed with the cosmid vector pHC79 have been described elsewhere (Manning et al, submitted).

Colony blotting with antiserum. Transformant colonies obtained in the cloning experiments were transferred to nitrocellulose filters and lysed in situ by the method of Henning et al. (17). Colonies producing the soluble hemag-

glutinin were detected with *E. coli*-absorbed rabbit antiserum followed by goat anti-rabbit immunoglobulin G coupled with horseradish peroxidase as described by Hawkes et al. (16).

Bal 31 nuclease digestion. Bal 31 nuclease was used to construct deletions basically according to the method described by Maniatis et al. (23).

Southern DNA hybridizations. Chromosomal fragments hybridizing with the cloned DNA were detected by the procedure of Southern (31) as described elsewhere (23).

Replicating colonies onto nitrocellulose filters. Colonies containing DNA hybridizing with the cloned DNA were detected by the procedure of Grunstein and Hogness (15) as described elsewhere (23).

Plasmid-encoded proteins in *E. coli* K-12 minicells. Minicells were purified on sucrose gradients, and the plasmid-encoded proteins were labeled with [^{35}S]methionine (1,270 Ci/mmol; Radiochemical Centre, Amersham, England) as described previously (1, 21). The proteins were visualized by autoradiography on Kodax X-Omat film after solubilization and running on sodium dodecyl sulfate-polyacrylamide gels as described previously (2), using a modification of the procedure of Lugtenberg et al. (22).

Electrophoretic protein blotting (Western blots). Protein blotting was performed as described by Towbin et al. (33).

Cell envelope preparations. Cell envelopes were prepared from a 10-ml shaken overnight culture by the small-scale method described previously (26).

RESULTS

The antiserum used in the following experiments was used in Western blot analysis with *V. cholerae*. In both classical and El Tor strains, a protein of about 24,000 daltons was detected as the major component capable of reacting with the antiserum. This protein could also be detected in cell envelope fractions (Fig. 1), which suggests that the protein may be a loosely cell-associated, probably peripheral protein. This is in agreement with the suggestion by Finkelstein et al. (9) that the protein could be cell associated in vivo. The observed size is slightly smaller than the 32,000 daltons previously described (10), but clearly this is the only protein in this size range with which the antiserum reacts. The small descrepancy may be due to differences in electrophoresis conditions. An additional band of about 70,000 daltons was detected in strain O17; the nature of this material is unknown. It could be another protein detected owing to not totally pure protein being used for raising the antiserum. Alternatively, it could represent a multimeric form of the smaller protein, which is detected owing to incomplete solubilization.

Detection of the clone. A gene bank of BamHI-cut DNA from *V. cholerae* 569B cloned into the BamHI site of pBR322 was screened by colony hybridization with antiserum to the soluble hemagglutinin by the procedure used previously (24, 32). Of about 3,000 colonies, 1 reacted with the antiserum. This colony together with several negatively reacting colonies was then purified and tested for hemagglutinating activity. The *E. coli* K-12 strain LE392, into which the various plasmids were introduced, was hemagglutination negative (Fig. 2). The colony which reacted positively with the antiserum could be reisolated with the antiserum and showed hemagglutination activity, whereas the other colonies showed no reaction. The plasmid in the positively reacting clone was designated pPM471. Transformants containing plasmid pPM471 DNA isolated after extraction and retransformation into strain LE392 were all hemagglutina-

FIG. 1. Western blot analysis of cell envelopes of *V. cholerae* 569B and O17 and *E. coli* K-12 LE392. The blots were developed with rabbit antiserum to the soluble hemagglutinin followed by goat anti-rabbit immunoglobulin G coupled with horseradish peroxidase.

tion positive, thus showing that the hemagglutinating capacity of the clone must be plasmid encoded. A hemagglutination titer comparable to that of *V. cholerae* was not obtained for two reasons. First, *V. cholerae* has several other hemagglutinins, whereas the clone has only one. Second, as will be seen below, this cloned gene is poorly expressed in *E. coli* K-12.

Sugar inhibition and RBC activity. Sugar inhibition tests were performed with the clone to confirm the identity of the hemagglutinin. Hemagglutination by the clone harboring pPM471 was not inhibited by any of the sugars tested, as compared with El Tor strain O17 which was inhibited by D-mannose and D-fructose. The clone also showed minimal if any hemagglutination activity when tested with human RBCs compared with maximum titers when mouse RBCs were used. This is in agreement with Finkelstein et al. (15), who showed that the soluble hemagglutinin unlike the major cell-associated hemagglutinins was not inhibited by any
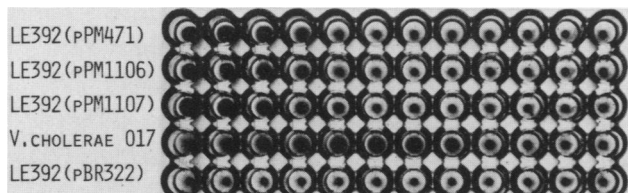

FIG. 2. Hemagglutination of mouse RBCs by *V. cholerae* O17 and the various *E. coli* K-12 derivatives. The wells contain twofold serial dilutions of bacteria starting with 5×10^9/ml in the left most well.
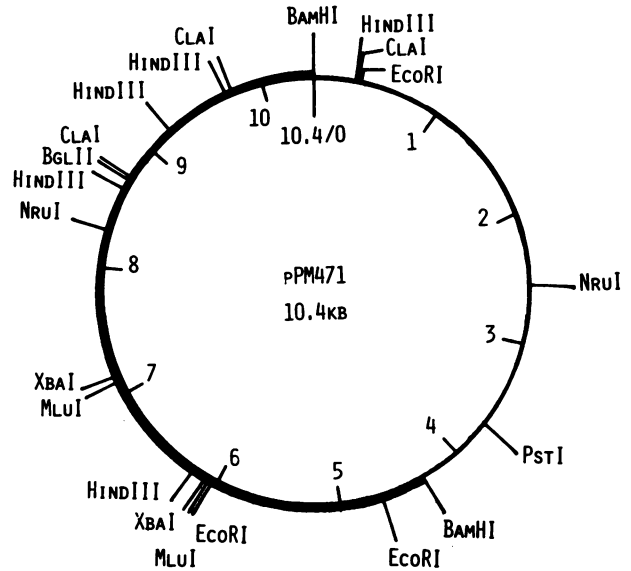

FIG. 3. Restriction endonuclease cleavage map of plasmid pPM471. The region of cloned *V. cholerae* DNA is shown by the thick line; the vector, pBR322, is shown by the thin line.

sugars tested so far and that the soluble hemagglutinin was markedly more active against mouse RBCs and minimally active against human RBCs.

Protease activity. The soluble hemagglutinin is reported to have protease activity (9). We tried to test this simply by streaking cultures onto casein agar plates and by incubating cell extracts of a strain hyperproducing the cloned protein (to be described elsewhere) in wells in the same plates. No protease activity could be detected.

Restriction analysis of pPM471. Plasmid pPM471 was digested with a range of restriction endonucleases, and a restriction map of the cloned DNA was constructed (Fig. 3). The cloned fragment of *V. cholerae* 569B DNA is 6.1 kb.

Deletion analysis of pPM471. As can be seen from the restriction map of pPM471 in Fig. 3, there are a number of conveniently spaced *Hin*dIII sites. Thus, by partially digesting the DNA with *Hin*dIII, diluting, and then ligating, it was possible to isolate plasmids in which one of the various *Hin*dIII fragments had been deleted. Strains harboring pPM1101, pPM1102, or pPM1103 (Fig. 4) were assayed for hemagglutination activity, and from such data it was clear that at least part of the gene coding for the activity must lie within the 2.35-kb *Hin*dIII fragment (coordinates 6.2 to 8.55 kb).

Subcloning of pPM471. The *Eco*RI-*Cla*I and the 2.35-kb *Hin*dIII fragments of pPM471 were cloned in plasmids pBR322 and pUC8, respectively, to give plasmids pPM1106 and pPM1108 (Fig. 4). Both of these subclones contained the gene as they expressed hemagglutination activity. The gene was further localized with the isolation from pPM1106 of plasid pPM1107. This plasmid was derived by deleting the *Mlu*I fragment (coordinates 6.1 to 7.1 kb on pPM471) from pPM1106. Plasmid pPM1107 still produced a hemagglutinin-positive phenotype and contained 1.15 kb of contiguous *V. cholerae* 569B DNA.

Generation of deletions with *Bal* 31 nuclease. To localize further the gene for the hemagglutinin, we used *Bal* 31 nuclease. This enzyme is a single-strand-specific nuclease which, by varying incubation times, can be used to produce
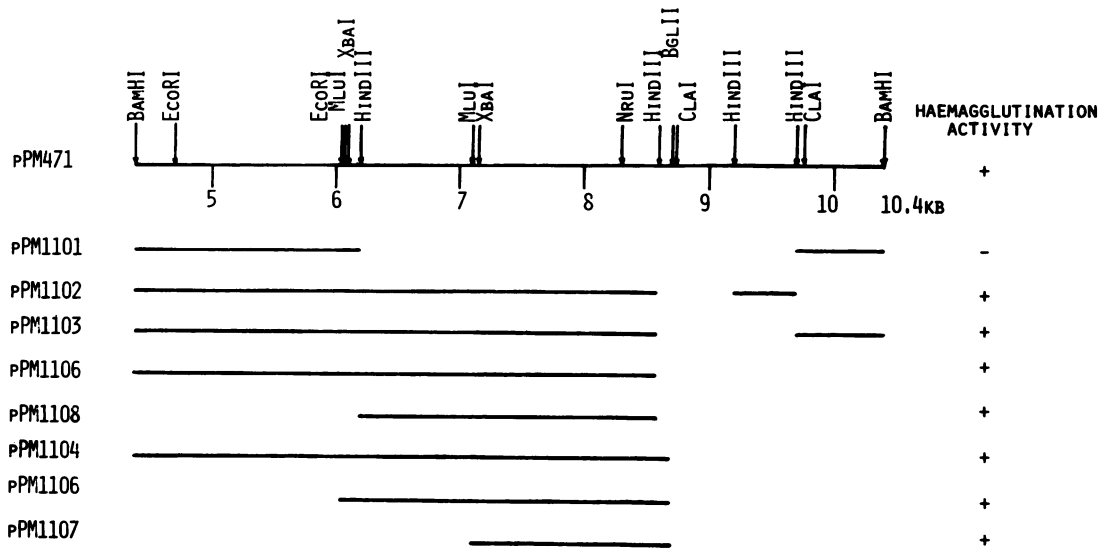
FIG. 4. Deletion analysis and subclones of pPM471. The lines indicate the regions of cloned DNA of plasmid pPM471 retained in the deletion or subcloned. Plasmid pPM1106 was derived by subcloning into pBR322, and pPM1108 was derived by subcloning into pUC8.

a family of deletions from a given restriction endonuclease cleavage site (Fig. 5). pPM1107 was digested with ClaI and then incubated in the presence of Bal 31 at 30°C. At various intervals samples were taken, and the reaction was stopped. ClaI linkers were added to enable the ends of the deletion to be defined precisely. The DNA was then transformed into E. coli K-12 LE392. The same procedure was repeated on pPM1107 DNA which had been digested with EcoRI, but EcoRI linkers were used. In this way a number of plasmids with various deletions extending into the cloned DNA from opposite ends were derived from pPM1107. Some of these are shown in Fig. 6. By assaying for hemagglutination activity, this allowed us to localize the gene to within a 0.72-kb fragment of DNA. Assuming a molecular weight of 110 for the average amino acid, this would give an upper limit of about 27,000 daltons for the hemagglutinin.

Analysis of plasmid-encoded proteins in E. coli K-12 minicells. Several of the plasmids were introduced into the minicell-producing E. coli DS410. Minicells were purified and labeled with [³⁵S]methionine, and the plasmid-encoded proteins were visualized by autoradiography after polyacrylamide gel electrophoresis. All and only those plasmids capable of conferring a hemagglutinin-positive phenotype

encoded a protein of 24,000 daltons (Fig. 6). Plasmid pPM1127 was the shortest Bal 31 derivative still capable of mediating hemagglutination. Interestingly, the hemagglutinin protein was not as well expressed (Fig. 6). This may be due to the fact that a pBR322 promoter which could amplify expression was also deleted or that its own promoter was damaged in the deletion process.

Southern hybridizations. As mentioned previously, a soluble hemagglutinin is found in both biotypes of V. cholerae. However, this has not been shown at a DNA level. To analyze for the presence of homologous DNA in the two biotypes, the HindIII fragment of pPM1106 was isolated and nick translated with [³²P]ATP. This was used to probe whole genomic DNA extracted from various V. cholerae strains which were digested with HindIII. This was also confirmed with the MluI-ClaI fragment of pPM1107.

A single band of approximately 2.35 kb was detected in each of the V. cholerae O1 strains, indicating that the DNA encoding for the hemagglutinin is conserved between the classical and El Tor biotypes.

Cloning of the hemagglutinin gene from El Tor biotype. A cosmid gene bank derived from V. cholerae O17 (El Tor, Ogawa) was constructed previously (Manning et al., submit-

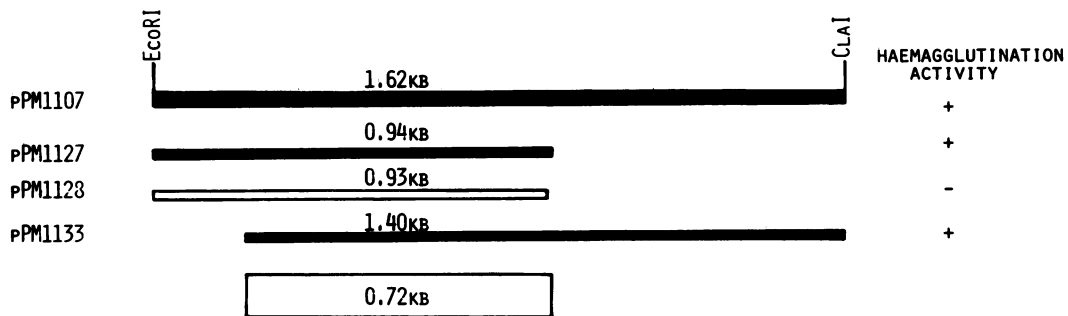FIG. 5. Bal 31 deletions of pPM1107. The solid lines represent the DNA present in plasmids which are capable of mediating hemagglutination. The open lines are hemagglutination negative. The box at the bottom is the maximum region of DNA available for encoding the gene for the hemagglutinin.
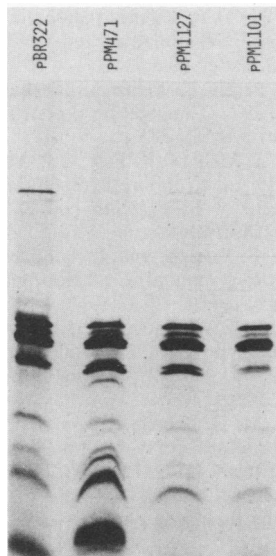
FIG. 6. Plasmid-encoded proteins in *E. coli* K-12 minicells. Minicells harboring the various plasmids were purified, labeled with [^{35}S]methionine, and solubilized in sodium dodecyl sulfate, and the plasmid-encoded proteins were visualized by polyacrylamide gel electrophoresis and autoradiography.

ted). From this bank, 500 colonies were patched onto nutrient agar containing ampicillin and then transferred onto nitrocellulose disks. These disks were then screened with the 6.1-kb *Bam*HI fragment of pPM471 labeled with ^{32}P. Several colonies reacted positively with the probe, and these were isolated and purified.

Cosmid DNA were extracted from the positively reacting clones and digested with various restriction endonucleases. Their DNA profiles were then compared with that of pPM471, and all positive clones were shown to have in common with pPM471 a 2.35-kb *Hind*III fragment.

DISCUSSION

Using antiserum to the soluble hemagglutinin, we detected, from our gene banks of *V. cholerae* classical strain 569B in *E. coli* K-12, a clone which reacts with the antiserum and also hemagglutinates mouse RBCs. The recombinant plasmid present in this clone was isolated and designated pPM471. Upon retransformation into a number of *E. coli* K-12 strains, the hemagglutination activity was also introduced, indicating that this activity is encoded on plasmid pPM471.

Restriction mapping showed that plasmid pPM471 contains a 6.1-kb *Bam*HI insert. By deletion analysis with both restriction enzymes and *Bal* 31 nuclease digestion, we were able to localize the gene for the soluble hemagglutinin to within 0.72 kb of DNA, suggesting an upper limit of 27,000 daltons for the protein. A size of 32,000 daltons has been previously reported (10). We detected in minicells a protein of 24,000 daltons correlating with hemagglutinating activity. Since it is an exported protein it would be expected to have a signal sequence of about 20 amino acids or 2,000 daltons, which would use up nearly all of the available coding capacity. This protein is identical to that which we detected in *V. cholerae* by Western blot analysis with antiserum to the purified protein. The gene for this protein was designated *sha*, for soluble hemagglutinin.

It has been reported previously that the soluble hemagglu-

tinin is present in both biotypes. This has been shown at a protein level, and we were interested to see whether DNA encoding for the soluble hemagglutinin was conserved between the classical and El Tor biotypes. By Southern DNA hybridizations we showed that there is DNA homology between a number of strains from both biotypes.

By radioactively labeling the cloned *Bam*HI fragment of *V. cholerae* DNA in pPM471, we were able to screen a cosmid bank which was derived from *V. cholerae* O17 (El Tor, Ogawa). Several positively reacting clones were detected and subsequently shown to share a 2.35-kb *Hind*III fragment in common with pPM471, implying that the gene encoding the *V. cholerae* soluble hemagglutinin was cloned from strains of both El Tor and classical biotypes. This will enable us to study the soluble hemagglutinin from both biotypes and to compare the degree of conservation.

It would seem that because of the nature of the properties of the soluble hemagglutinin it may play a direct role in the pathogenesis of disease. It has been suggested that it is involved in nicking and activation of the cholera enterotoxin (5). It may also act as an adherence factor in allowing *V. cholerae* to attach to the intestinal epithelium by virtue of its protease activity which could help in penetration of the mucous layer (9). We were unable to detect protease activity for the protein described here; however, this may reflect specificity in its substrate range rather than lack of activity. Tests with the specific substrates fibronectin, ovomucin, and lactoferrin are still to be performed. However, since protease activity is a fundamental property of the soluble hemagglutinin, we cannot at this stage confirm the identity of it and the hemagglutinin described here.

We propose to anlyze the various properties attributed to this protein by introducing into *V. cholerae* specific mutations in the hemagglutinin gene to examine the effects on the pathogenesis of cholera by such strains, and to look at the specific molecules which may be affected, such as cholera toxin.

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