Molecular Cloning of a Gene Coding for a *Vibrio cholerae* Haemagglutinin

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Recombinant plasmids encoding a *Vibrio cholerae* haemagglutinin were isolated from the highly virulent *V. cholerae* strain C5 by cosmid cloning. Both *Escherichia coli* HB101 containing the recombinant plasmids and *V. cholerae* C5 were able to agglutinate a variety of erythrocytes from human and animal origin; this haemagglutination was not inhibited by D-mannose or L-fucose. Subcloning of the recombinant cosmid DNA revealed that a 1.3 kb DNA fragment was sufficient for haemagglutinin production in *E. coli* HB101. Under direction of this 1.3 kb *Vibrio* DNA fragment, two proteins were made in *E. coli* minicells, of 27 and 10 kDa. Haemagglutinin-encoding sequences were not detected in every *V. cholerae* strain.

INTRODUCTION

The production of cholera toxin is the major virulence factor in infectious diarrhoea caused by *Vibrio cholerae*. Toxin production, however, is not the only virulence factor; several others are known to play a role in the colonization of the small intestine by this organism. These factors include motility, chemotaxis and the synthesis of extracellular DNAase and proteinases, which enable the bacteria to penetrate the mucus of the small bowel. One or more types of adhesins are thought to be involved in the attachment of the bacteria to the mucus and/or the brush borders of the epithelial cells of the intestine.

Recently, many efforts have been made to purify and characterize the bacterial surface components that are involved in the adhesion of *V. cholerae* to the intestinal epithelium. Several surface components have been suggested to play a role in this process, but until now, none of these components has been incontrovertibly identified as a crucial adhesive factor. The suggested components include LPS (Chitnis *et al.*, 1982), proteins of the flagellar sheath (Hranitzky *et al.*, 1980; Attridge & Rowley, 1983) and haemagglutinins (Jones *et al.*, 1976; Jones & Freter, 1976). Haemagglutinins function as adhesins in various types of enterotoxigenic bacteria, such as enterotoxigenic *Escherichia coli* strains of animals and human origin (reviewed by Jones, 1977; Duguid & Old, 1980; Gaastra & De Graaf, 1982).

V. cholerae haemagglutinins are less well documented than those of the enterotoxigenic *E. coli* strains, but several experiments indicate that they must be considered as virulence factors (Finkelstein *et al.*, 1983; Booth *et al.*, 1984; Guinée *et al.*, 1985) that might mediate the attachment of *V. cholerae* to the epithelial cell surface (Jones *et al.*, 1976; Jones & Freter, 1976).

Based on the inhibitory effect of L-fucose and D-mannose in the haemagglutination assay, four types of haemagglutinin have been distinguished by Hanne & Finkelstein (1982). Only one of these types, a haemagglutinin that occurs both bound to the *Vibrio* cell surface and excreted into the culture medium, has been purified (Finkelstein *et al.*, 1978; Chaicumpa & Atthasishtha, 1979; Finkelstein & Hanne, 1982; Svennerholm *et al.*, 1983). In this paper, we report on the molecular cloning of a gene coding for a *V. cholerae* haemagglutinin.

METHODS

Bacterial strains, plasmids and culture conditions. Strains of *V. cholerae* and *E. coli* described in this paper are listed in Table 1. Also shown in this Table are the plasmids used for cloning of *V. cholerae* DNA. Bacteria were

Table 1. *Strains and plasmids used*

Strain	Characteristics	Reference/source
<i>E. coli</i> ED8767	<i>supE supF hsdS</i> [Res ⁻ Mod ⁻ (k)] <i>met recA56</i>	Murray <i>et al.</i> (1977)
<i>E. coli</i> HB101	F ⁻ <i>hsdS20</i> [Res ⁻ Mod ⁻ (B)] <i>recA13</i> <i>ara-14 proA2 lacY1 xyl-5 rpsL20</i> (Sm ^r) <i>supE44</i> λ ⁻	Boyer <i>et al.</i> (1969)
<i>E. coli</i> DS410	F ⁻ <i>met minA minB rpsL</i>	Dougan & Sherratt (1977)
<i>V. cholerae</i> C5	Serotype Ogawa, biotype El Tor	Guinée <i>et al.</i> (1985); RIVM*
<i>Vibrio</i> C8	Non-O1 <i>Vibrio</i> , <i>tox</i>	RIVM*
<i>V. cholerae</i> C14	Serotype Inaba, biotype classical	RIVM*
<i>V. cholerae</i> C31	Serotype Ogawa, biotype El Tor	RIVM*
<i>V. cholerae</i> C41	Serotype Ogawa, biotype El Tor†	RIVM*
<i>V. cholerae</i> 395	Serotype Ogawa, biotype classical	R. B. Sack‡
Plasmids		
pTCF	Ap ^r , contains <i>cos</i> -site of λ	Grosveld <i>et al.</i> (1982)
pUC19	Ap ^r , contains polylinker in <i>lacZ'</i>	Yanisch-Perron <i>et al.</i> (1985)
pACYC184	Cm ^r Tc ^r	Chang & Cohen (1978)

* Field isolate belonging to the collection of the Rijksinstituut voor Volksgezondheid en Milieuhygiëne (RIVM), Bilthoven, The Netherlands.

† Spontaneous avirulent mutant of strain C5.

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grown in Brain-Heart Infusion broth (BHI) or Luria-Bertani broth (LB), containing the appropriate antibiotics (ampicillin, 100 μ g ml⁻¹; chloramphenicol, 35 μ g ml⁻¹; tetracycline, 12.5 μ g ml⁻¹), at 30 °C or 37 °C in a reciprocating water bath shaker (200 r.p.m.).

Cosmid cloning of *V. cholerae* DNA. High *M_r* chromosomal DNA of *V. cholerae* C5 was prepared according to the method described by Van Embden *et al.* (1983). Batches containing 50–100 μ g DNA were partially digested to varying degrees with the restriction enzyme *Sau* 3A. Three batches of digested DNA with mean fragment sizes of more than 50 kb, 30–50 kb and less than 30 kb were pooled. This pooled digested DNA was layered on top of a 10–40% (w/v) sucrose gradient in 1 M-NaCl, 5 mM-EDTA, 20 mM-Tris/HCl, pH 8.0. After centrifugation for 21 h at 75000 *g* and 20 °C, 1.2 ml fractions of the gradient were collected. Fractions containing DNA fragments of 30–45 kb were pooled, dialysed against 10 mM-Tris/HCl, 1 mM-EDTA, pH 8.0 (TE-buffer), precipitated with ethanol and dissolved in TE-buffer to a concentration of 1 mg ml⁻¹.

This size-fractionated, digested *Vibrio* DNA was ligated to the *Bam*HI site of the cosmid vector pTCF according to the method of Ish-Horowitz & Burke (1981). This *Bam*HI site is flanked by *Sal*I sites, which enable excision of the cloned DNA from the vector by digestion with *Sal*I. Briefly, left and right arms of vector DNA were obtained by digestion of pTCF with either *Hpa*I or *Eco*RI, followed by treatment with alkaline phosphatase and digestion with *Bam*HI. *Hpa*I/*Bam*HI-digested pTCF (0.3 μ g), *Eco*RI/*Bam*HI-digested pTCF (0.3 μ g) and the 30–45 kb *Vibrio* DNA fragments obtained by digestion with *Sau*3A (1 μ g) were mixed in 5 μ l ligation buffer (20 mM-Tris/HCl, 10 mM-MgCl₂, 10 mM-dithioerythritol, 1 mM-ATP, pH 7.6). This mixture was incubated with T4 DNA ligase (1 Weiss unit; BioLabs) for 2 h at 15 °C. The ligated DNA was packaged in bacteriophage λ heads and tails according to the procedure of Grosveld *et al.* (1981). (Double-digested vector DNA and packaging extracts were a gift of Dr G. Grosveld, University of Rotterdam, The Netherlands.) Phages were diluted in 100 mM-NaCl, 10 mM-MgCl₂, 10 mM-Tris/HCl, pH 7.5 and used to transduce stationary phase *E. coli* strain ED8767, grown in LB medium containing 0.4% maltose and 10 mM-MgCl₂. Phage suspension (0.1 ml) and bacterial culture (0.2 ml) were mixed, incubated at room temperature for 15 min and then incubated for 30 min at 37 °C. Portions of this transduction mixture were plated on LB plates containing ampicillin (0.1 ml per plate). Twenty thousand ampicillin-resistant colonies were obtained per μ g *Vibrio* DNA. One thousand colonies were grown separately in wells of microtitre trays containing LB medium, mixed with glycerol (final concentration 20%, v/v) and stored at –70 °C until further analysis.

Subcloning of recombinant DNA from the cosmid clones. Fragments of recombinant DNA contained in the cosmid vector pTCF were subcloned in the plasmid vectors pACYC184 and pUC19. To that end, cosmid DNA was digested with the appropriate restriction enzymes, electrophoresed in a 0.6% agarose gel and the fragments to be subcloned were recovered from the gel by electro-elution. Vectors were digested with suitable restriction enzymes and treated with alkaline phosphatase. Vector DNA (200 ng in 10 μ l ligation buffer) was ligated to a twofold molar excess of the DNA fragment to be subcloned, for 2 h at room temperature. For circularization of DNA fragments, 40 ng of the desired fragments were incubated overnight in 0.1 ml ligation buffer at 18 °C. Transformation of competent bacteria was done according to the method of Cohen *et al.* (1973) as modified by Dagert & Ehrlich (1979).

Purification of plasmid DNA, digestion with restriction enzymes and electrophoresis. Plasmid DNA was purified as described by Birnboim & Doly (1979), in some cases followed by CsCl-equilibrium centrifugation. DNA was digested with restriction enzymes under the conditions recommended by the suppliers. Dephosphorylation of digested DNA with calf intestine alkaline phosphatase (Boehringer) was done as described by Maniatis *et al.* (1982). DNA was electrophoresed in 0.6% agarose gels in TBE-buffer (90 mM-Tris, 90 mM-boric acid, 2.5 mM-EDTA, pH 8.3) at 10 V cm⁻¹ or in E-buffer (35 mM-Tris, 35 mM-NaH₂PO₄, 1 mM-EDTA, pH 7.6) at 2 V cm⁻¹.

Haemagglutination assay. Bacteria used for haemagglutination assays were grown overnight in LB broth at 30 °C in a reciprocating water bath shaker (200 r.p.m.). Bacteria were harvested by centrifugation, washed once with KRT-buffer (0.125 M-NaCl, 5 mM-KCl, 1.3 mM-MgSO₄, 2.75 mM-CaCl₂, 10 mM-Tris/HCl, pH 7.0) and suspended in KRTM (KRT-buffer containing 0.5% (w/v) D-mannose) to an OD₆₆₀ of 10. Blood was collected in Alsever's solution (32.5 mM-trisodium citrate, 77 mM-NaCl, 2%, w/v, dextrose) and washed three times with KRT-buffer. Suspensions of 1% (v/v) erythrocytes were prepared by carefully pipetting 1 ml of packed blood cells into 99 ml KRTM. For the haemagglutination assay, a 0.1 ml portion of the bacterial suspension was serially twofold-diluted with KRTM in the wells of a microtitre tray; equal volumes of the erythrocyte suspension were added and the assay was left for 1 h at ambient temperature. Titres were expressed as the reciprocal of the highest dilution of the bacterial suspension that still gave visible haemagglutination.

Labelling of proteins produced in *E. coli* minicells and electrophoresis on SDS-polyacrylamide gradient gels. Plasmids were introduced in the minicell-producing *E. coli* strain DS410 (Dougan & Sherratt, 1977). Isolation of minicells from overnight cultures and labelling of proteins with L-[³⁵S]methionine (1040 Ci mmol⁻¹, 50 µCi per incubation; 1 Ci = 37 GBq) or a mixture of tritiated L-amino acids (50 µCi per incubation) were as described by Mooi *et al.* (1981). Proteins were analysed by electrophoresis of lysates of the labelled minicells on 24 cm long 12–20% (w/v) polyacrylamide gradient gels containing 0.1% (w/v) SDS (Mooi *et al.*, 1981). Labelled products were visualized after autoradiography or fluorography on Kodak XAR-5 film at –70 °C.

Southern blotting of DNA and hybridization with radioactively labelled DNA probes. Digested DNA was electrophoresed overnight at 2 V cm⁻¹ in E-buffer (35 mM-Tris, 35 mM-NaH₂PO₄, 1 mM-EDTA, pH 7.6). After staining and photographing, the gel was soaked in 0.5 M-NaOH, 1.5 M-NaCl (45 min), next in 1.0 M-Tris/HCl, 3.0 M-NaCl, pH 6.0 (45 min), and finally the DNA was transferred to a nitrocellulose membrane (Schleicher and Schüll; 0.45 µm) as described by Southern (1975). The DNA probe used for hybridization was labelled with [α -³²P]dATP (3000 Ci mmol⁻¹, 25 µCi per incubation) by nick-translation as described by Van Dongen *et al.* (1981). Labelled probes (10⁶ c.p.m.) were hybridized to the membrane-bound DNA in 25 ml of a solution containing 5 µg sheared calf thymus DNA ml⁻¹, 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 30% (v/v) deionized formamide, 10% (w/v) dextran sulphate, 0.3 M-NaCl, 30 mM-trisodium citrate and 20 mM-sodium phosphate, pH 6.8 (Van Dongen *et al.*, 1981). After hybridization (16 h, 55 °C), membranes were washed three times with 2 × SSC (1 × SSC is 0.15 M-NaCl, 0.015 M-trisodium citrate, pH 7.0) at room temperature and twice with 0.1 × SSC at 50 °C, and autoradiographed at –70 °C.

RESULTS

Isolation of cosmid clones that produce a V. cholerae haemagglutinin

The *V. cholerae* E1 Tor strain C5 was chosen as a source of DNA for cosmid cloning. This strain is highly virulent in a rabbit infection model (Guinée *et al.*, 1985). Unlike most other *Vibrio* strains, strain C5 exhibited only one type of haemagglutinating activity, whatever the growth conditions. This activity could not be inhibited with D-mannose or L-fucose (MFRHA) and occurred with various types of erythrocytes (Table 2). After partial digestion of the DNA of this strain with the restriction enzyme *Sau* 3A, 30–45 kb fragments were isolated, ligated to the *Bam*HI site of cosmid vector pTCF and cloned in *E. coli* strain ED8767 by a cosmid-cloning procedure; 1000 of the resulting clones were grown up separately. The recombinant plasmids of 20 of these clones were isolated and the sizes of the plasmids were estimated by agarose gel electrophoresis. All 20 clones contained plasmids with sizes between 40 and 53 kb. As the complexity of the DNA of Gram-negative bacteria is 4–5 × 10³ kb (De Ley, 1971), each recombinant plasmid contained approximately 0.8–1.0% of the *Vibrio* genome.

Six hundred clones were screened for the expression of a haemagglutinating activity that could not be inhibited with L-fucose and D-mannose. Three clones were isolated that had the ability to agglutinate human erythrocytes in the presence of 0.5% D-mannose and L-fucose. The haemagglutination titres obtained with these clones were low (2²–2³) compared to the titre obtained with *V. cholerae* C5 grown under similar conditions (2⁸–2⁹). In order to study if the expression of the *Vibrio* haemagglutinin in *E. coli* was host-strain dependent, the recombinant

Table 2. *Haemagglutinating activities of V. cholerae strain C5 and of E. coli clones containing Vibrio DNA*

Titres were obtained after agglutination of 1% (v/v) suspensions of erythrocytes by serially twofold-diluted bacterial suspensions (highest concentration: 10 OD₆₆₀ units per ml) in the presence of 0.5% D-mannose. Titres are expressed as the reciprocal of the highest dilution that still gave visible haemagglutination. The assay was done twice (three times for the clones carrying pVC hag-232 and -235) with different batches of bacteria. Most results were fully reproducible; some varied within one titre unit. In the latter case, the lowest titre is shown in the Table.

Strain	Titre with erythrocytes of:						
	Chicken	Human	Guinea pig	Pig	Horse	Sheep	Mouse
<i>V. cholerae</i> C5	128	256	512	512	512	512	512
<i>E. coli</i> HB101(pACYC184)	0	0	0	2	0	0	0
<i>E. coli</i> HB101(pVC hag-1)*	2	4	4	8	8	8	8
<i>E. coli</i> HB101(pVC hag-11)	2	4	4	4	4	8	8
<i>E. coli</i> HB101(pVC hag-232)	4	8	8	8	8	16	16
<i>E. coli</i> HB101(pVC hag-235)	2	0	0	2	0	2	2

* Haemagglutinating activities of *E. coli* HB101 containing pVC hag-2 or pVC hag-3 were the same as for the clone carrying pVC hag-1.

plasmids were isolated from the haemagglutinin-producing clones and introduced into several *E. coli* K12 strains. No host-dependent differences in expression of haemagglutinating activity were found (results not shown). Table 2 shows the haemagglutination titre obtained with one of the clones, containing the recombinant plasmid pVC hag-1 in *E. coli* HB101, with various types of erythrocytes. It appeared that all types of erythrocytes that were agglutinated by *V. cholerae* C5 were also agglutinated by this clone. Both for *V. cholerae* C5 and for *E. coli* HB101 (pVC hag-1) the lowest haemagglutination titres were obtained with chicken and human erythrocytes; the titres obtained with horse, pig, sheep, mouse and guinea pig erythrocytes were more or less equal. The presence of the haemagglutinin-encoding plasmids in the clones also caused the bacteria to clump together (autoagglutination). Autoagglutination could not be seen with the host strain, *E. coli* HB101, nor with *V. cholerae* C5.

The recombinant plasmids were isolated from the three clones and restriction maps of these plasmids, named pVC hag-1, -2 and -3, were constructed (Fig. 1). A strong homology in the restriction patterns of the three recombinant plasmids appeared to exist over a length of 17 kb (Fig. 1, region I), indicating that they might contain overlapping segments of the *V. cholerae* genome.

The restriction pattern of pVC hag-2 DNA was almost completely overlapping with that of pVC hag-3, except for a region of approximately 6 kb that was present in pVC hag-2, but absent in pVC hag-3 (Fig. 2, region II). A stretch of DNA with a restriction pattern similar to region II DNA on pVC hag-2 was also present in pVC hag-1, but its localization was different (Fig. 1). Southern blot hybridizations with pVC hag-101, which contains part of the region II DNA (Fig. 2a), as a labelled probe, indicated that region II DNA did not originate from the genome of *V. cholerae* C5, but from the chromosomal DNA of the host bacterium, *E. coli* ED8767, used for cloning (results not shown). It probably represents a transposable element that was inserted into the recombinant DNA after transduction.

Localization of the genetic information for haemagglutinin production

The three recombinant plasmids that contain the genetic information for a *V. cholerae* haemagglutinin shared an overlapping region of 17 kb (Fig. 1, region I). The haemagglutinin-encoded sequences were localized more precisely by deletion mapping and subcloning of purified restriction fragments of pVC hag-1 and -2 in the plasmid vectors pACYC184 and pUC19 (Fig. 2). Of the resulting recombinant plasmids (pVC hag-10, -11, -21, -23, -27 and -28; Fig. 2a and b), only pVC hag-11, -21 and -23 were able to generate haemagglutinating activity in *E. coli* HB101. More subclones were isolated by constructing deletion mutants of pVC hag-23,

Fig. 1. Restriction enzyme cleavage maps of recombinant cosmids containing genetic information for production of a *V. cholerae* haemagglutinin. The maps were constructed by double and triple digestions of the recombinant DNA and by Southern blot hybridizations of digested DNA with purified restriction fragments. Thin lines represent insert *V. cholerae* C5 DNA; thick lines represent DNA of the cosmid vector pTCF. Recombinant DNA fragments are aligned to show the homology in the restriction patterns of the inserts. Region I indicates the overlapping region present in all three cloned DNA fragments. Region II represents the transposon-like insert in pVC hag-1 and -2. Restriction sites are shown for *Bam*HI (B), *Hpa*I (H), *Kpn*I (K), *Nru*I (N), *Sal*I (S), *Sst*I (Ss), *Xba*I (Xb) and *Xho*I (X).

using the *Bam*HI and *Xba*I sites that are present both in the insert DNA and in the polylinker of the vector DNA. All recombinant plasmids obtained in this way (pVC hag-231, -232 and -234; Fig 2*b*) expressed haemagglutinating activity in *E. coli* HB101. The smallest of these plasmids, pVC hag-232, contained only 1.3 kb of *Vibrio* DNA. Plasmid pVC hag-235 was constructed by digestion of the insert of pVC hag-232 with *Nru*I and ligation of the resulting *Xba*I/*Nru*I fragment into pACYC184 (Fig. 2*b*). *E. coli* HB101 (pVC hag-235) was able to agglutinate chicken, mouse and sheep erythrocytes, but with a very low titre (Table 2) compared to the clone carrying pVC hag-232. The autoagglutination of *E. coli* HB101 (pVC hag-235) was also slower than that of the clone carrying pVC hag-232. Probably, the 0.8 kb DNA insert of pVC hag-235 still could express the haemagglutinin, but was unable to generate full haemagglutinating activity in *E. coli* HB101.

Identification of proteins expressed by haemagglutinin-encoding plasmids

A fluorograph of tritiated proteins made by *E. coli* minicells containing pVC hag-231 and -232 and separated on a 12–20% SDS polyacrylamide gradient gel is shown in Fig. 3. In addition to the vector-encoded β -lactamase and its precursor, only two polypeptides, with apparent sizes of 27 and 10 kDa, were encoded by pVC hag-232 (lane *b*), the smallest recombinant plasmid that generated full haemagglutinating activity in *E. coli* HB101. These two polypeptides probably represent full-size proteins and are not truncated products of genes that are only partially present in the DNA of pVC hag-232, since these polypeptides were also produced in clones carrying pVC hag-11 (not shown) and pVC hag-231, which have much larger inserts. The 10 kDa protein was not synthesized in minicells carrying pVC hag-235. The labelling of the 10 kDa protein appeared to be more intense in minicells carrying pVC hag-232 than in those carrying pVC hag-231. The reason for this might be found in the location of the strong *lacZ'* promoter, but final conclusions have to await sequencing data.

Occurrence of haemagglutinin-encoding sequences in other *V. cholerae* strains

The 6.4 kb *Bam*HI insert of pVC hag-11, which contains the genetic information for haemagglutinin production, was used as a radioactive probe for hybridization with Southern blots of *Bam*HI-digested *Vibrio* DNA and *Bam*HI/*Sal*I double-digested recombinant plasmid DNA. As shown in Fig. 4, this probe hybridized with the 6.4 kb *Bam*HI fragments of pVC hag-1 and -3 and with the 4.2 kb *Bam*HI/*Sal*I fragment of pVC hag-2 (pVC hag-2 contains only part of the 6.4 kb *Bam*HI fragment proximal to the *Sal*I site of the vector). This indicated that the

Fig. 2. Recombinant plasmids made by deletion or by subcloning of pVC hag-1 (a) and pVC hag-2 (b). The recombinant plasmids were constructed as follows. Plasmids pVC hag-10 and -11 were obtained by ligation of the 17 and 6.4 kb *Bam*HI fragments of pVC hag-1 into the *Bam*HI site of pACYC184. Plasmid pVC hag-101 was made by cloning the 3.3 kb *Xho*I fragment in the *Sal*I site of pUC19. Plasmid pVC hag-21 was derived from pVC hag-2 after deletion of the 22.5 kb *Kpn*I fragment. Plasmids pVC hag-23, -27 and -28 were constructed by ligation of the 13.8 kb *Sal*I fragment, the 11.6 kb *Sst*I fragment and the 10.4 kb *Xba*I fragment of pVC hag-2 to *Sal*I-, *Sst*I- and *Xba*I-digested pUC19, respectively. Plasmid pVC hag-234 was made by deletion of the 10 kb *Xba*I fragment of pVC hag-23. Plasmids pVC hag-231 and -232 were constructed by using the *Bam*HI and *Xba*I sites next to the *Sal*I site in the polylinker of pUC19. These recombinants were made by digestion of pVC hag-23 with *Bam*HI and *Xba*I, respectively, and recircularization of the vector containing restriction fragments. By cloning the 0.8 kb *Xba*I/*Nru*I fragment of pVC hag-232 into *Xba*I/*Nru*I double-digested pACYC184, pVC hag-235 was obtained. Thin lines represent insert DNA; thick lines represent vector DNA. The position and orientation of promoters for the tetracycline-resistance gene in pACYC184 and for the *lacZ'* gene in pUC19 are indicated by arrows, when tested. Abbreviations for restriction sites are the same as in Fig. 1, but not all sites shown in Fig. 1 are given in this figure. The ability to induce mannose-resistant haemagglutination (MRHA) in *E. coli* HB101 is indicated on the right side.

corresponding arrangement of the restriction sites in the three cloned fragments indeed resulted from sequence homology.

A 6.4 kb fragment hybridizing with the probe could also be detected in *Bam*HI-digested chromosomal DNA of *V. cholerae* strains C5 (which served as a source of the DNA used in cosmid cloning), C14, C41 and 395 (Fig. 4, lanes e, g, i and j, respectively). Such a hybridizing fragment could not be visualized amongst the *Bam*HI fragments of the DNA from *E. coli* ED8767 (lane d) and *Vibrio* strain C8, a non-toxicogenic, non-O1 *Vibrio* strain that is unable to colonize the intestine (lane f). Also, in the digested DNA of the virulent *V. cholerae* strain C31 this fragment could not be found (lane h).

Fig. 3. Fluorograph of an SDS-containing 12–20% polyacrylamide gradient gel showing tritiated proteins made by *E. coli* minicells under the direction of cloned *V. cholerae* DNA. The minicells of *E. coli* DS410 contained pUC19 (lane *a*), pVC hag-232 in pUC19 (lane *b*) or pVC hag-231 in pUC19 (lane *c*). Each lane contained 30000–60000 c.p.m. The 27 and 10 kDa proteins are indicated by asterisks next to lane *b*. The positions of β -lactamase (L) and its precursor (pL), and of various marker proteins (sizes in kDa) are indicated.

DISCUSSION

This paper describes the isolation of *E. coli* clones containing *V. cholerae* DNA coding for a mannose- and fucose-resistant haemagglutinin. The *V. cholerae* strain C5, which was used as a source of the DNA, is a highly virulent strain: it very effectively colonizes the small intestine of rabbits and less than 10^3 c.f.u. were able to provoke cholera in the DISC-model developed by Guinée *et al.* (1985). A correlation between haemagglutination and infectivity has been demonstrated.

Although most *V. cholerae* strains exhibit several types of haemagglutinating activity, only the mannose- and fucose-resistant type could be detected with strain C5. From the cosmid library of *Vibrio* DNA in *E. coli*, three clones with overlapping DNA inserts (out of 600 tested) were isolated that exhibited this type of haemagglutination. However, the haemagglutination titres obtained with the *E. coli* clones carrying the recombinant plasmids were invariably low (2^1 – 2^4) compared to those of *V. cholerae* C5 (2^7 – 2^9). The reason for this low level of expression is as yet unknown. Several factors might be involved, such as transcription, translation, transport, the exposure of the haemagglutinin on the *E. coli* surface, or a combination of these factors. A positive regulator of transcription, the *toxR* gene product (Miller & Mekalanos, 1984), was found to have no influence on the haemagglutinating activity of the clones (results not shown).

Except for the lower titres obtained with the clones, both the clones and *V. cholerae* C5 behaved similarly in the haemagglutination-typing experiment described in Table 2: the seven types of erythrocytes tested in this experiment were agglutinated in the presence of D-mannose by strain C5 and by the clones, and strain C5 as well as the clones had lower haemagglutination titres with chicken and human erythrocytes than with the other types.

Fig. 4. Southern blot hybridization of digested cosmid DNA and *V. cholerae* chromosomal DNA with radioactively labelled pVC hag-11 DNA. Cosmid DNA (50 ng) was double-digested with *Bam*HI and *Sal*I, and 5 µg of *V. cholerae* or *E. coli* chromosomal DNA was digested with *Bam*HI. Fragments were separated by electrophoresis in a 0.8% agarose gel, transferred to a nitrocellulose membrane and hybridized with 32 P-labelled pVC hag-11 DNA. The hybridization profiles are shown for *Bam*HI/*Sal*I-digested DNA of pVC hag-1 (lane a), -2 (lane b) and -3 (lane c) and for *Bam*HI-digested chromosomal DNA of *V. cholerae* strains C5 (lane e), C14 (lane g), C31 (lane h), C41 (lane i) and 395 (lane j), and the non-O1 *Vibrio* strain C8 (lane f) and *E. coli* ED8767 (lane d). The labelled band near the top of the gel in lane a is a partially digested DNA fragment.

Subcloning of the inserts contained in the recombinant cosmids revealed that a DNA fragment of not more than 1.3 kb (pVC hag-232) was sufficient for haemagglutinin production in *E. coli* HB101. Only two proteins were encoded by a pVC hag-232 in minicells, with apparent sizes of 27 and 10 kDa. With sheep and mouse erythrocytes, a slight but consistently measured haemagglutination could be detected with the clone containing the 0.8 kb *Xba*I/*Nru*I fragment (pVC hag-235), but the titre was 2^2 – 2^3 lower than obtained with the clone containing pVC hag-232. Probably, the haemagglutinin is still synthesized in the clone carrying pVC hag-235, but some factor necessary for efficient expression of haemagglutinating activity is lacking. This factor might be the 10 kDa protein: synthesis of this protein could not be detected in minicells carrying pVC hag-235. Further investigations will be necessary to determine if both proteins are involved in the expression of haemagglutinating activity; these investigations will include sequence analysis of the 1.3 kb insert of pVC hag-232 and site-directed mutagenesis.

Until now, only one of the four types of *V. cholerae* haemagglutinin has been purified, viz. a haemagglutinin with an activity that could not be inhibited with D-mannose or L-fucose, that could be found both in a cell-associated and in an excreted form and that possesses proteolytic activity (Finkelstein & Hanne, 1982; Finkelstein *et al.*, 1983; Svennerholm *et al.*, 1983). As the size of the haemagglutinin purified by Finkelstein, as determined by SDS-PAGE, differed substantially from those of the proteins synthesized in minicells under direction of pVC hag-232, we have no reason to assume that the haemagglutinin encoded by pVC hag-232 is similar to the one isolated from *V. cholerae* (Finkelstein & Hanne, 1982). However, we have not tested our cloned haemagglutinin for proteolytic activity, nor for cross-reactivity with sera raised against the purified haemagglutinin.

There are no indications that the haemagglutinating activity of *V. cholerae* C5 and of the respective *E. coli* clones is caused by the production of fimbriae, as is the case with several

enterotoxigenic *E. coli* strains (Gaastra & De Graaf, 1982). First, we could never detect any fimbriae by electron microscopy, neither on *V. cholerae* C5, nor on *E. coli* HB101 carrying the haemagglutinin-encoding plasmids. Second, in all cases studied so far, the genetic information necessary for fimbriae production consists of at least five genes, comprising a DNA fragment of more than 5 kb; the smallest fragment coding for *V. cholerae* haemagglutinin is only 1.3 kb and not more than two proteins are expressed from this fragment.

Although it has been suggested that *V. cholerae* haemagglutinins are involved in the adhesion of the bacteria to the brush borders of the intestinal epithelium (Jones *et al.*, 1976; Jones & Freter, 1976), we have no evidence that the cloned haemagglutinin functions as an adhesin. Adhesion assays with rabbit intestinal epithelial cells yielded non-conclusive results, but one of the reasons for this might be the low level of expression of haemagglutinating activity in the *E. coli* clones.

As shown in the Southern blotting experiment, sequences hybridizing to the haemagglutinin-encoding insert of pVC hag-11 could not be detected in all *V. cholerae* strains. They appeared to be absent in the non-O1 *Vibrio* strain C8, and also in the *V. cholerae* El Tor strain C31. Yet, stationary-phase cells of both strains expressed a weak mannose-resistant haemagglutinating activity, which points to the existence of a second type of haemagglutinin with a similar activity. As we could only isolate one type of haemagglutinin-encoding sequence from the cosmid library of C5 DNA, either this second type is not present in strain C5, or this haemagglutinin is not expressed by the clones in the cosmid library. Studies to test a possible role of the haemagglutinin in the infection process are in progress.

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