Expression of foreign epitopes in CS3 fimbriae

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In the name of Allah

ABSTRACT

Cell appendages including fimbriae have been used for the expression of foreign antigenic determinants and therefore have been considered as potential immunogens in vaccine development. Two virulence factors, fimbriae and toxins, are involved in pathogenesis of Enterotoxigenic *E. coli*. Fimbriae are colonization factors that have been used for immunization in farm animals (as vaccine) and human volunteers in vaccine development studies. Toxins, heat stable (ST) and heat labile (LT), induce secretion of water and electrolytes in the intestine resulting in diarrhoea. LT is immunogenic whereas ST which is a small peptide is not. Considerable efforts have been applied to increase the immunogenicity of ST for inclusion in vaccine against ETEC by coupling the toxin to carrier molecules through chemical and genetic procedures.

The aims of this study were:

-To develop the CS3 pili of CFA/II$^+$ of enterotoxigenic *E. coli* as a delivery system for the expression of foreign antigenic determinants to the bacterial cell surface.

-To make an immunogenic ST toxoid as a step towards a vaccine against Enterotoxigenic *E. coli*.

CS3 is expressed by most strains bearing colonization factor antigen II (CFA/II) and has been shown to have a constant structure among CS3 expressing strains. Cloning and sequencing of the *cstH* gene, encoding the major fimbrial subunit of CS3, from 6 strains from different geographical region showed a high degree of similarity at both nucleotide and amino acid levels. A panel of 19 Mabs against CS3 were isolated and characterized. These Mabs and two Mabs from previous work have been used to study the antigenic variation in CS3. 20 Mabs had identical reactivity with CS3 from 5 strains, but Mab 11:2 failed to recognize the CS3 pili on strain E248750-1 whereas it reacted with its denatured form in immunoblot. The same results were obtained with *E. coli* K-12 harbouring a recombinant

CS3 operon which including the *cstH* gene from this strain. These results suggest that there is some antigenic variation in CS3 in different strains and that Mab 11:2 recognizes a conformational epitope, including aa 39, which is different in CstH from E248750-1 (Asn→Lys).

These Mabs were also used for epitope analysis of CS3. Two sets of nested deletions from C- and N-terminal of the *cstH* were generated and truncated genes fused to the *phoA* gene lacking both promoter and signal sequence. These fusion proteins and hybrid CstH::ST and CstH::LT-B_{44-64} were expressed in *E. coli* K-12, and by immunoblot analysis using the Mabs, some epitopes were characterized.

Two exposed regions of *cstH* have been used for insertion and expression of the foreign epitopes. These epitopes were derived from the B subunit of LT (linear epitope) and ST toxin(non-linear epitope). PCR mutagenesis and amplification was employed to introduce two unique sites into *cstH* and generating the *est* and *eltB* epitope cassettes respectively. These foreign epitopes were inserted in-frame into the two unique sites of the mutants *cstH* gene.

Protein analysis of the various constructs in the T7 RNA polymerase/promoter expression system in *E. coli* K12 by immunobloting using antibodies to both carrier and insert, demonstrated the expression of the hybrid proteins.

The ability of the hybrid pili to be expressed on the call surface was demonstrated by immuno dot blot analysis and immunofluorescence microscopy. The hybrid *cstH* genes were reinserted into the *cst* operon and the hybrid gene clusters were expressed in *E. coli* K12. Immunofluorescence microscopy using Mabs against CstH and ST showed the expression of the hybrid CS3 pili on the bacterial surface.

The immunogenicity of the constructs was evaluated in mice. The plasmids encoding hybrid CS3 pili were transferred into *Salmonella typhimurium* vaccine strain G30 and expression of the hybrid pili was confirmed by immunofluorescence microscopy. The sera

from orally and intraperitoneally immunized mice with hybrid CS3 were able to recognize CS3 in the ELISA.

The experiments which have been reported in this thesis have been indicated that the CS3 pili can tolerate the insertion of the complex peptides into at least two internal regions. This system therefore has the potential for delivery of foreign epitopes which may be useful in vaccine development.

Statement

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text.

I give consent to this copy of my thesis, when deposited in the University library, being available for loan and photocopying.

Bagher Yakhchali

February 1996

I wish to dedicate this thesis to my wife

In the memory of the 20th anniversary of my parents

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I must thank GOD who gave me the opportunity to become familiar with another aspect of his creation; molecular pathogenesis of microorganisms. I would also like to acknowledge the receipt of a scholarship from the Ministry of Culture and Higher Education of the Islamic Republic of IRAN.

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Abbreviations

Ap	ampicillin
ATP	adenosine 5'-triphosphate
bp	base pair
BSA	bovine serum albumin
CIP	calf intestinal phosphatase
Cm	chloramphenicol
GTP	guanosine 5'-triphosphate
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylene-diamine-tetra-acetic-acid, disodium salt
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
kb	kilobase
kDa	kilodalton
Km	kanamycin
kV	kilovolts
LB	Luria broth
LPS	lipopolysaccharide
MAb	monoclonal antibody
mg	milligram
ml	millilitre

mM	millimolar
MSHA	D-mannose-sensitive haemagglutinin
MRHA	D-mannose-resistant haemagglutination
NA	nutrient agar
NB	nutrient broth
OD	optical density
OMP	outer membrane protein
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pmol	picomoles
Pmx	polymyxin B
Rif	rifampicin
RNA	ribonucleic acid
RT	room temperature
R	resistant
S	sensitive
SDS	sodium dodecyl sulphate
TBS	Tris-buffered saline
Tc	tetracycline
TCP	toxin-coregulated pilus
TTBS	Tris-buffered saline with Tween-20 added
UTP	uridine 5'triphosphate
μF	microFarad
μg	microgram

µl	microlitre
µm	micrometre
UV	ultraviolet
V. cholerae	*Vibrio cholerae*
v/v	volume per volume
w/v	weight per volume
aa	amino acid
CFA	colonization factor agar.
ddNTP	dideoxyribonucleoside triphosphate
EtBr	ethidium bromide
g	gram
g/l	grams/litre
IPTG	Iso-propyl b-D-thiogalactopyranosid
Klenow	Klenow fragment of *E. coli* DNA polymerase I
mA	milliAmps
µg	microgram
ml	microlitre
nt	nucleotide
r.p.m	revolutions per minute
TEMED	N,N,N',N'- tetramethylenediamine
X-gal	5-bromo-4-chloro-3-indoyl-b-d-galacto-pyranoside
X-pho	5-bromo-4-chloro-3-indolylphosphate
r	recombinant
r *cst*	recombinant *cst* operon either containingheterologous gene or hybrid gene
PHD	Profile network prediction HeiDelberg

LT	Heat-Lable toxin
LT-B	Heat-Lable toxin B subunit
ST	Heat-Stable toxin
E. coli	*Escherichia coli*
ETEC	Enterotoxigenic *E. coli*

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General Introduction

1.1 Introduction

Escherichia coli is one of the most common microorganisms in the normal flora of the intestinal tract of humans and animals. Sixty years after its discovery by Theodor Escherich (1885), the relationship between *E. coli* and diarrhoea was established. However, it is also associated with extraintestinal disease in humans such as infections of the urinary tract, neonatal meningitis and septicemia (Viboud, 1995).

Pathogenic *E. coli* belong to a limited number of O:H serotypes (Ørskov *et al.*, 1977). Diarrhoea causing *E. coli* have been classified into five major categories, based on virulence properties, interaction with the intestinal mucosa, clinical syndromes, epidemiology and O:H serotypes (Donnenberg and Kaper, 1992; Levine, 1987) as given below:

Enterotoxigenic E. coli (ETEC) are a major cause of infant and travellers' diarrhoea.

Entroinvasive E. coli (EIEC) are a cause of dysentery.

Enteropathogenic E. coli (EPEC) are involved in infant diarrhoea.

Enterohaemorrhagic E. coli (EHEC) are a cause of haemorrhagic colitis and haemolytic uremic syndrome.

Enteroaggregative E. coli (EAggEC) are associated with prolonged diarrhoea.

The common features of these groups are the ability to adhere to the intestinal brush border and the production of enterotoxins or cytotoxins.

This thesis involves research into the development of the CS3 (*c*oli *s*urface antigen *3*) subcomponent of CFA/II of human enterotoxigenic *E. coli* (ETEC) as a delivery system for foreign antigens (epitopes). A description of the fimbriae and toxins of ETEC and the delivery systems for the expression of foreign antigenic determinants will be made here. The progress in vaccine development against ETEC diarrhoea has also been examined.

1.2 Enterotoxigenic *E. coli* (ETEC)

Travellers' diarrhoea is usually caused by ingestion of contaminated food or water and enterotoxigenic *E. coli* (ETEC) are the most commonly isolated pathogens. (Black, 1986; 1993; Levine, 1990; Taylor and Echeverria, 1986). ETEC are also the major cause of diarrhoea in developing countries (Black *et al.*,1981) and are responsible for considerable morbidity and mortality among children of endemic regions (Black, 1993). ETEC cause 50 million diarrhoeal cases and about 800,000 deaths annually (Black, 1986). In the farm industry, ETEC cause severe diarrhoea in neonatal and young herd animals (Gyles, 1971; Gyles *et al.*, 1974; Gross *et al.*, 1978; Guinee *et al.*, 1977; Smith and Linggood, 1972). The serotypes and fimbrial types of human isolates are distinct from those of animal ETEC and indicate species-specific characteristics (Soderlind and Mollby, 1979).

Two virulence factors are involved in the pathogenesis of ETEC. Fimbriae or pili are host-specific and mediate the colonization of the bacteria to the epithelial surface of the small intestine. Following colonization of the intestine by the bacteria, enterotoxins are elaborated. These can be either heat-labile (LT) or heat stable toxins (ST).

1.3 Enterotoxins

Two types of toxins (ST and LT) either alone or together, are produced by the ETEC strains isolated from both humans and animals. These toxins are usually plasmid-encoded and cause accumulation of water and electrolytes in the small intestine resulting in diarrhoea (Sherman *et al.,* 1972; Smith and Linggood, 1971).

1.3.1 ST family of toxins

Two types of ST toxin, STa (or STI) and STb (or STII) are produced by ETEC. These are structurally, functionally, immunologically and genetically distinct from each other (Burgess *et al.,* 1978; Gyles, 1992). STa is a small peptide with a size of approximately 2 kDa. It resists heat up to 100°C for 15 min., is soluble in water and organic solvents and is resistant to proteolytic enzymes such as pronase, trypsin and chymotrypsin (Alderet and Robertson, 1978; Smith and Halls, 1967). It is methanol soluble and is active in the infant mouse gut (Whipp *et al.,* 1981).

1.3.1.1 Genetics of the ST family

STs are encoded by genes designated *est* and four *est* genes have been cloned from ETEC. The gene $estA_1$ encoding STIa (or STp) from a plasmid of bovine ETEC strain, B41, was cloned and sequenced (So and McCarthy, 1980) and shown to be part of a transposon, Tn1681. The gene $estA_2$ was cloned and sequenced from a human ETEC strain (CRL 25090) isolated in Bangladesh (De Wilde *et al.,* 1981). The gene $estA_3$ was cloned and sequenced (Moseley *et al.,* 1983; Stieglitz *et al.,* 1988) from a human ETEC

strain isolated in Bangladesh. The *estA*$_4$ allele was cloned and sequenced [from an 80 MDa plasmid of a human ETEC strain, J5-4, isolated in Mexico (Stieglitz *et al.,* 1988)]. Guzman-Verduzco and Kupersztoch (1989) resequenced *estA*$_2$ and *estA*$_3$ genes and have shown that *estA*$_3$ and *estA*$_4$ are identical genes and that the C-terminus of *estA*$_2$ was not the same as previously reported. These sequence data have suggested that the *estA*$_2$ and *estA*$_3$ (*estA*$_4$) genes are very similar whereas *estA*$_1$ has the least homology with the others (Guzman-Verduzco and Kupersztoch, 1989; Stieglitz *et al.,* 1988). The *estA* genes are very rich in A+T content (>65%) (Stieglitz *et al.,* 1988) and it has been suggested that *estA* may have originated from another organism with high A+T content DNA (Dallas, 1990).

1.3.1.2 Structure of ST toxins

Based on the nucleotide sequence data and properties of purified STs, the STa toxin is divided into two classes, STp (STA_1) from porcine and STh (STA_2, STA_3, and STA_4) from human strains of ETEC. The STp (STA_1) is a peptide of 18 amino acids (Okamoto and Takahara, 1990; So and McCarthy, 1980; Takao *et al.,* 1983) and the STh (STA_2, STA_3 and STA_4) 19 amino acid residues (Aimoto *et al.,* 1982; Guzman-Verduzco and Kupersztoch, 1989; Mosely *et al.,* 1983; Stieglitz *et al.,* 1988). The STAs are extracellular peptides which are synthesized as a 72 amino acid precursor, Pre-Pro-ST (Okamoto and Takahara, 1990; Stieglitz *et al.,* 1988). The Pre domain consists of 19 amino acids showing the characteristics of a typical signal sequence (von Heijne, 1985) which is cleaved by signal peptidase I (Guzman-Verduzco *et al.,* 1989) to produce a 53 amino acid Pro-ST protein. This is translocated across the inner membrane to the periplasm (Guzman-Verduzco and Kupersztoch, 1990). Pro-ST is then secreted into the

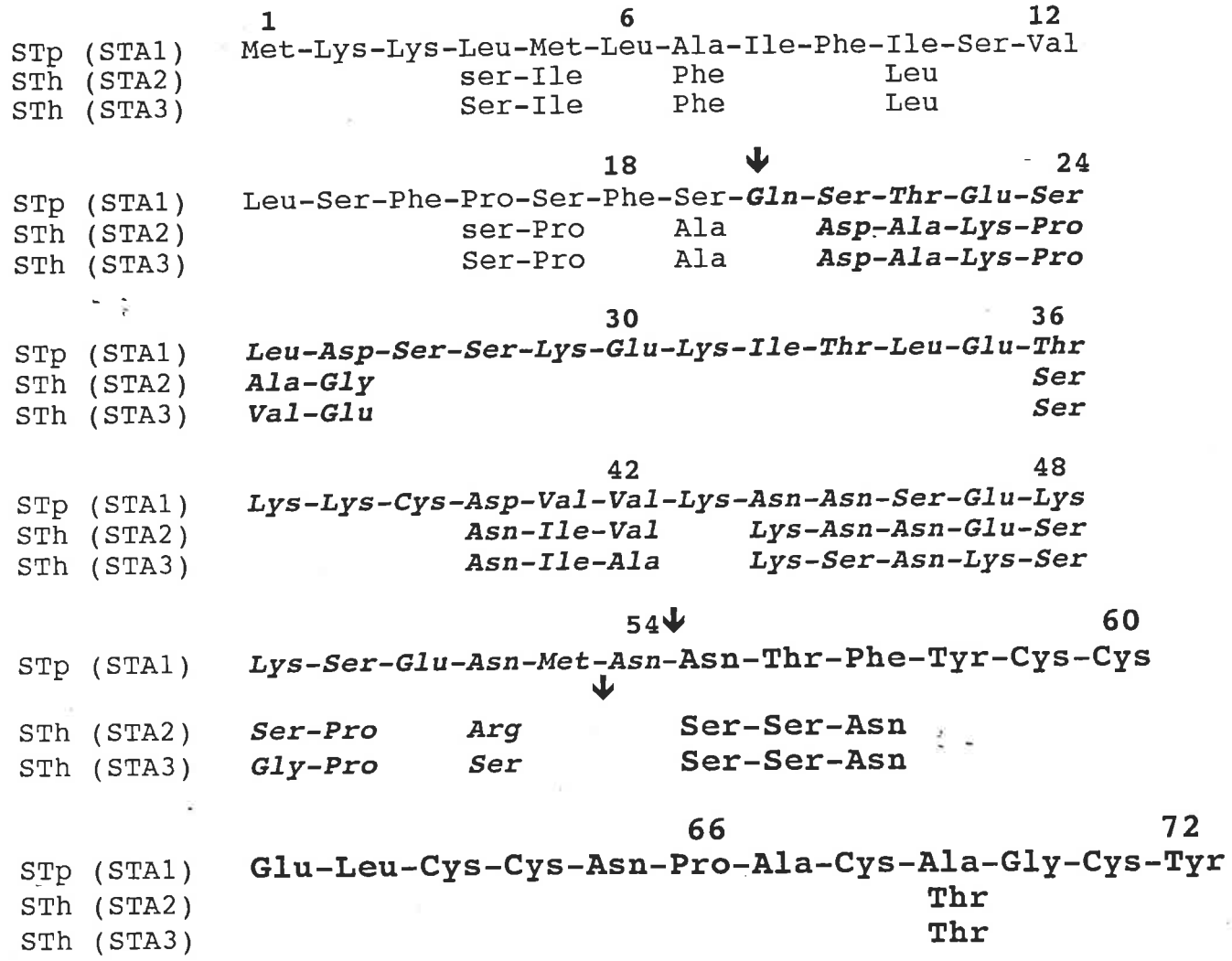
environment and is cleaved between the Met_{53} and Asn_{54} for STA_2, STA_3, STA_4 and between Asn_{54} and Ser_{55} for STA_1 to yield the mature STa toxins of 19 or 18 amino acids, respectively (Rasheed *et al.,* 1990; Stiegltiz *et al.,* 1988, Yang *et al.,* 1992) (Fig 1.1). The Pre-domain which has signal sequence properties is essential for translocation of the Pro-ST through the inner-membrane and its deletion leads to intracellular degradation of ST (Yang *et al.,* 1992). The function of the Pro-region is not well understood, but it is apparently not essential for export of ST to the culture, as evidenced by the secretion of ST into the culture medium by the Pro-deleted mutants (Yang *et al.,* 1992). Sanchez *et al.,* (1993) showed that in-frame fusion of mature ST to the signal sequence of the B subunit of LT (LT-B) resulted in synthesis and secretion of ST, which implied that the size and conformation of ST allowed the molecule to exit through the outer membrane.

Yamanaka *et al.,* (1994) suggested that the Pro-domain is involved in determining the correct pattern of disulfide bond formation. The substitution of Cys_{39} within this domain caused a significant decrease in ST activity in the culture supernatant, the accumulation of inactive ST in the periplasmic space, and an alteration in the cleavage site of the intermediate of STp. Thus it was concluded that Cys_{39} was important for recognition by the processing enzymes.

Several structurally homologous and antigenically cross-reacting STa proteins are produced by different enteric bacteria. These include STp (STA_1) from porcine (Takao *et al.,* 1983) and STh (STA_2, STA_3, and STA_4) from human strains of ETEC; NAG-ST from *Vibrio cholerae* non-O1 (Artia *et al.,* 1986; Takao *et al.,* 1985; Yoshimura *et al.,* 1986), H-ST from *V. cholerae* non-O1 Hakata strain (Artia *et al.,* 1991b), M-ST from *V. mimicus* (Artia *et al.,* 1991a), Y-ST from invasive *Yersinia enterocolitica* (Pai *et al.,*

Fig 1.1 Comparison of STa amino acid sequences.

Deduced amino acid sequence for the full-length pre-pro-STA (72 amino acids) is shown. Arrows show the cleavage sites for STp (Ser_{19}-Gln_{20} and Asn_{54}-Asn_{55}) and STh (Ser_{19}-Gln_{20} and Met_{53}-Asn_{54}). The STp sequence is from So and McCarthy (1980) and the STh (STA2 and STA3) are from Guzman-Verduzco and Kupersztoch (1989). For STh, only amino acids which are different from STp are shown. Identical amino acids are empty spaces.


1
6
12
STp (STA1) Met-Lys-Lys-Leu-Met-Leu-Ala-Ile-Phe-Ile-Ser-Val
STh (STA2) ser-Ile Phe Leu
STh (STA3) Ser-Ile Phe Leu
18
24
STp (STA1) Leu-Ser-Phe-Pro-Ser-Phe-Ser-Gln-Ser-Thr-Glu-Ser
STh (STA2) ser-Pro Ala Asp-Ala-Lys-Pro
STh (STA3) Ser-Pro Ala Asp-Ala-Lys-Pro
30
36
STp (STA1) Leu-Asp-Ser-Ser-Lys-Glu-Lys-Ile-Thr-Leu-Glu-Thr
STh (STA2) Ala-Gly Ser
STh (STA3) Val-Glu Ser
42
48
STp (STA1) Lys-Lys-Cys-Asp-Val-Val-Lys-Asn-Asn-Ser-Glu-Lys
STh (STA2) Asn-Ile-Val Lys-Asn-Asn-Glu-Ser
STh (STA3) Asn-Ile-Ala Lys-Ser-Asn-Lys-Ser
54
60
STp (STA1) Lys-Ser-Glu-Asn-Met-Asn-Asn-Thr-Phe-Tyr-Cys-Cys
STh (STA2) Ser-Pro Arg Ser-Ser-Asn
STh (STA3) Gly-Pro Ser Ser-Ser-Asn
66
72
STp (STA1) Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Ala-Gly-Cys-Tyr
STh (STA2) Thr
STh (STA3) Thr

1978, Takao *et al.*,1984; 1985) and noninvasive *Yersinia enterocolitica* (Robins-Browne *et al.,* 1993), C-ST from *Citrobacter freundii* (Guarino *et al.,* 1987; 1989a), O1-ST from a cholera toxin gene-positive strain of *V.cholerae* O1 (Takeda *et al.,* 1991; Yoshimo *et al.,* 1993), EAST1 from Enteroaggregative *E. coli* (Savarino *et al.,* 1993b) and ST from *Klebsiella pneumoniae* (Guarino *et al.,* 1989b). These STs have common biological properties and share antigenic determinants with *E. coli* ST (Okamoto *et al.,* 1981; 1983; Takao *et al.,* 1985). They have a common highly conserved region of 10 amino acids including 6 cysteine residues in the same corresponding positions (Shiminoshi *et al.,* 1987).

STh and STp share a highly conserved C-terminal 13 amino acids (aa 5-18 in STp and 6-19 in STh) which is antigenic, as well as containing receptor binding and enterotoxic properties (Gariepy and Schoolnik, 1986; Yoshimura *et al.,* 1985). Epitope analysis of STh using 4 monoclonal antibodies (Mab) showed that there are three epitopes in this region. Amino acids Leu_9 and Tyr_{19} could be recognized by two distinct Mabs, Thr_{16} and Tyr_{19} are associated with an epitope for another Mab. The data from this study also suggested that the N-terminal residues (Asn-Ser-Ser-Asn-Thr), thought not to be essential for the biological activity of STh, possess an important epitope (Asn_4 and Thr_5) which can induce neutralizing antibody (Takeda *et al.,* 1993). The C-terminal 13 amino acids conserved region contains 6 cysteine residues that participate in the formation of three disulfide bonds to form the tertiary structure of the toxin (Okamoto *et al.,* 1987). A periplasmic protein, termed DsbA (a disulfide bond epimerase), has been found to catalyze the formation of the disulfide bonds of STp as well as many other proteins in *E. coli* (Yamanaka *et al.,* 1994). All three disulfide linkages are critical for the conformational structure and are required for full expression of the biological activity of the toxin. Dissociation of the disulfide bonds results in a dramatic loss of ST activity

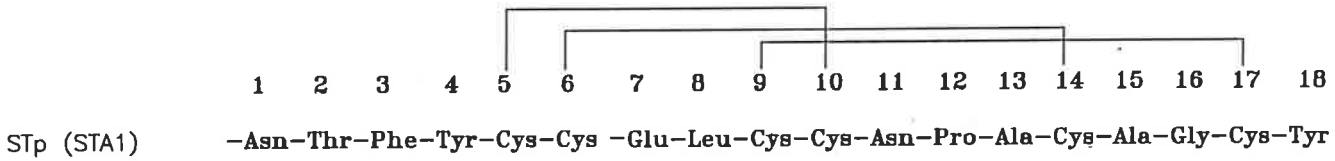
(Greenberg *et al.,* 1983; Okamoto *et al.,* 1987; Staples *et al.,* 1980; Yoshimura *et al.,* 1985). These issues were investigated through the chemical synthesis of analogues of the 13 amino acid C-terminal domain of STh and STp and substitution of residues. A pattern of disulfide linkage has been proposed (Fig 1.2), where Cys_7 is paired with Cys_{15}, Cys_6 with Cys_{11} and Cys_{10} with Cys_{18} (Gariepy *et al.,* 1987). This pattern is identical to that determined for STp (Ozaki *et al.,* 1991) and *Y. enterocolitica* and *V. cholerae* non-O1 ST (Shimonishi *et al.,* 1987), suggesting that all these toxins have similar conformation.

The contribution of each disulfide bond to the potency of the toxin is not equivalent. The Cys_7 and Cys_{15} are the most crucial pair for activity, followed by Cys_6 and Cys_{11}. The least important linkage is Cys_{10} and Cys_{18} but they still make a significant contribution to maximal potency. The folding pattern suggested for STh consists of three turns which are stabilized by the three disulfide bridges (Gariepy *et al.,* 1987). Study of the crystal structure of a 13 amino acid toxic domain analogue of STp revealed that the molecule has a right-hand spiral peptide backbone. Three β-turns are located along this spiral and fixed tightly by three intramolecular disulfide bonds (Ozoki *et al.,* 1991). The β-turn structures are located along the spiral from Cys_6-Cys_9, Asn_{11}-Cys_{14} and Cys_{14}-Cys_{17} of STp. The second turn is more important in the expression of the toxicity as suggested by the finding that the amino acids in the first and third β-turn can be replaced without affecting toxicity (Ozaki *et al.,* 1991). The contribution of individual amino acids to the biological activity of ST has been studied and shown that Asn_{11} of STp plays an essential role in the enterotoxic activity of the toxin. The amide group and the length of the side chain of Asn_{11} may be important for enterotoxic activity (Okamoto *et al.,* 1988). Replacement of Ala_{13} in the second turn of STp by other amino acids resulted in marked reduction in the toxic activity and binding to its receptor in rat

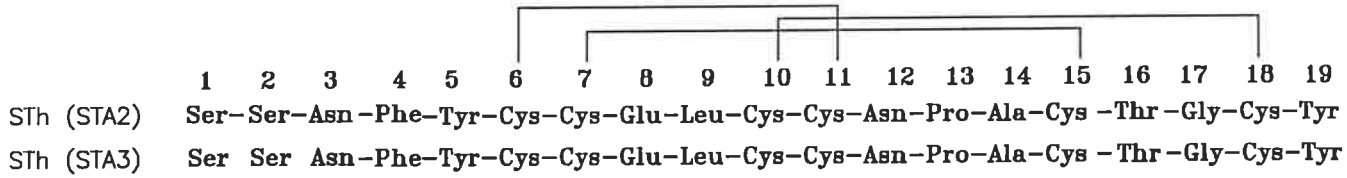
Fig 1.2 Amino acid sequences of STp and STh.

The amino acid sequences of the STp (panel A) and STh (panel B) are shown. The lines show a pattern of disulfide linkage in the molecule as suggested by Gariepy *et al.* (1987) and Ozaki *et al.* (1991).

A


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
STp (STA1) -Asn-Thr-Phe-Tyr-Cys-Cys -Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Ala-Gly-Cys-Tyr

B


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
STh (STA2) Ser-Ser-Asn-Phe-Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys -Thr-Gly-Cys-Tyr
STh (STA3) Ser Ser Asn-Phe-Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys -Thr-Gly-Cys-Tyr

intestinal cell membranes. Thus, it has been proposed that the second β-turn of the toxin molecule is important for the interaction of the toxin with its receptor protein. Takashi *et al.* (1994) have studied the three dimensional structures of analogues of STp by replacing Ala_{13} with Gly or Leu and concluded that the decrease and loss of the biological activity in these peptide analogues are because Ala_{13} (Ala_{14} in STh) is a putative receptor-binding residue.

1.3.1.3 Receptors for ST toxins

The presence of STa receptors has been demonstrated in the human small intestine and colon (Cohen *et al.,* 1988), rabbit caecum (Rao *et al.,* 1980), rat colon and ileum (Mezoff *et al.,* 1992), the human intestinal cell line CaCo-2 (Cohen *et al.,* 1993), and in the human colonic cell line T84 (Guarino *et al.,* 1988).

The STa receptor appears to be a protein or glycoprotein (Dreyfus and Robertson, 1984; Hirayama *et al.,* 1992, Vaandrager *et al.,* 1993). STa binds to the receptor through the reaction of a disulfide bond in STa with a sulphydryl group in the receptor, as suggested by the finding that STa could readily be dissociated from its receptor by treatment with dithiothreitol (Dreyfus and Robertson, 1984). Two classes of ST receptors, low-affinity and high-affinity, have been identified in intestinal membranes (Dreyfus *et al.,* 1984; Dreyfus and Robertson, 1984; Hugues *et al.,* 1991; Kuno *et al.,* 1985; Thompson, 1987). High-affinity receptors are not coupled to activation of particulate guanylate cyclase (Crane *et al.,* 1992; Dreyfus *et al.,* 1983) but the low-affinity receptors are associated with its activation (Crane *et al.,* 1992).

The analysis of labelled ST cross-linked to receptors by SDS-PAGE showed that there are several structural forms of the ST receptor: non-reducable 160, 136, 78, 71 and 56 kDa binding subunits and 160 and 136 kDa proteins which are able to be reduced to 78 and 71 kDa subunits, respectively (Kuno *et al.,* 1985; Ivans *et al.,* 1990). Active ST receptor was purified by affinity chromatography from the membrane lipid-bilayer of rat intestinal mucosa (Hugues *et al.,* 1992) and from the cytoskeleton-associated component of rat intestinal membranes (Hakki *et al.,* 1993). Analysis of affinity purified ST receptor from lipid-bilayer by SDS-PAGE and cross linking of labelled ST demonstrated a major protein subunit of 74 kDa and also 164 and 45 kDa subunits, confirming the structural heterogeneity of ST receptors. The purified protein did not possess guanylate cyclase activity which could be attributed to the instability of the enzyme during purification procedures (Hugues *et al.,* 1992).

The ST receptor isolated from rat cytoskeleton possessed a subunit size of 56 kDa which exhibited both high and low-affinity binding sites for ST. This ST binding protein was not coupled with particulate guanylate cyclase activity (Hakki *et al.,* 1993). These data suggest that ST binds to several different proteins in the intestinal membrane, but some of them do not have guanylate cyclase activity (Hakki *et al.,* 1993; Hugues *et al.,* 1992; Kuno *et al.,* 1985; Waldman *et al.,* 1985).

ST receptors are also heterogenous in their localization within intestinal brush borders. 40% of the total ST receptors seems to be associated with the lipid bilayer and can be extracted with detergents (Waldman *et al.,* 1985). The remaining insoluble receptors appear to be linked to the cytoskeleton of brush border membranes. The ST receptor is not coupled to particulate guanylate cyclase in detergent extracts but

cytoskeleton linked ST receptor is associated with guanylate cyclase (Kuno *et al.,* 1985; Waldman *et al.,* 1985).

Genes encoding ST receptor have been cloned from rat and human intestinal cDNA libraries (de Sauvage *et al.,* 1991; 1992; Schulz *et al.,* 1990; Singh *et al.,* 1991). A ST receptor of 121 kDa was predicted based on the deduced amino acid sequence. The cloned protein, termed Guanylyl cyclase-C (GC-C), which has ST binding and guanylate cyclase activity, contains an extracellular domain, peptide-binding region, a single, short trans-membrane domain, and a cytoplasmic region containing protein tyrosine kinase and guanylate cyclase catalytic domains.

The cloned GC-C gene encodes a new member of the particulate guanylate cyclase peptide receptor family associated with the cytoskeleton of the intestinal cells. Transfection of Cos-7 cells with the cloned ST receptor gene expressed particulate guanylate cyclase activity and the ability to specifically bind to ST with low affinity leading to accumulation of cyclic GMP (Schulz *et al.,* 1990). Transfection of Cos-7 and other mammalian cells with the human homologue GC-C exhibited similar results (de Sauvage *et al.,* 1991; 1992; Singh *et al.,* 1991). Vanderager *et al.* (1993) expressed GC-C in human embryonic 293 cells. Cyclic GMP concentrations in the cell were increased 40-fold in response to 1μM STa. The data indicated that GC-C is an N-linked glycoprotein.

In contrast to the studies of crude and purified ST receptor, which attribute the guanylate cyclase activity and ST binding to separate molecules in intestinal cells (Crane *et al.,* 1992; Hugues *et al.,* 1991; 1992; Hakki *et al.,* 1992; 1993; Ivans *et al.,* 1990; Katawa *et al.,* 1991; Waldman *et al.,* 1985), cloning and expression of the rat and human intestinal ST receptors suggest that the ST binding and particulate guanylate cyclase

activity are on a single transmembrane protein (de Sauvage *et al.,* 1991; 1992; Schulz *et al.,* 1990; Singh *et al.,* 1991). Comparison of receptors for ST in two intestinal cell lines, the human colon carcinoma cell line CaCo-2 and the rat intestinal cell line IEC-6 showed that CaCo-2 cells respond to ST with increased guanylate cyclase activity. Cloning and expression studies confirmed that the receptor present in CaCo-2 cells is a homologue of guanylyl cyclase (GC-C). Northern and PCR analysis indicated that guanylyl cyclase C is not expressed in IEC-6 cells, suggesting the presence of a different ST receptor that is not coupled to guanylyl cyclase activity (Mann *et al.,* 1993).

Recently, Visweswariah *et al.* (1994) purified a ST receptor from T84, a cell line derived from a human colonic carcinoma, with a size of 160 kDa. The purified receptor retained guanylyl cyclase activity.

Further detailed studies of purified and cloned receptor proteins may solve the discrepancy in these data. This includes the development of procedures which allow the purification of sufficient amounts of native ST receptor for comparisons to be made in the activity and amino acid sequences of cloned and native ST receptors.

1.3.1.4 Mechanism of action of ST

The initial step in the biological activity of STa involves binding of the toxin to the specific receptor on the intestinal epithelial cell membrane (Cohen *et al.,* 1987; Dreyfus and Robertson 1984; Kuno *et al.,* 1986) leading to the activation of particulate guanylate cyclase. Activated guanylate cyclase converts GTP to cyclic GMP (cGMP) resulting in accumulation of the cGMP in the intestinal epitelial cell (Field *et al.,* 1987; Huott *et al.,* 1988; Waldman *et al.,* 1986). This increase in cGMP mediates the

inhibition of sodium chloride absorption and stimulates chloride secretion with the concomitant elevation of water and electrolytes in the gut and consequently diarrhoea (Dreyfus and Robertson, 1984; Field *et al.,* 1987; Hughes *et al.,* 1978; Huott *et al.,* 1988; Kuno *et al.,* 1986; Waldman *et al.,* 1986; Waldman and Murad, 1987).

1.3.2 LT toxin family

Enterotoxigenic *E. coli* and *Vibrio cholerae* produce a heat-labile enterotoxin which belongs to a family of structurally and immunologically related proteins that cause diarrhoea in humans and animals (Holmes *et al.,* 1990). Heat-labile enterotoxins are composed of a single A polypeptide bound to a pentamer of the B polypeptide (Gill, 1976; Gill *et al.,* 1981; Sixma *et al.,* 1991).

The *E. coli-V. cholerae* enterotoxin family consists of two serogroups (Finkelstein *et al.,* 1987; Pickett *et al.,* 1988). Serogroup one comprises type I *E. coli* enterotoxin (LT-I) including LTh-I and LTp-I produced by human and porcine strains, cholera toxin (CT) and antigenically related enterotoxins from several other Gram negative enteric bacteria (Calva *et al.,* 1989; Prasad *et al.,* 1990; Rose *et al.,* 1989). Serogroup two consists of Type II *E. coli* enterotoxin (LT-II) including LT-IIa and LT-IIb (Pickett *et al.,* 1986; 1989; Pickett and Holmes, 1990). Antisera against LT-I or CT neutralize the other Type I toxins (Guth *et al.,* 1986; Pickett *et al.,* 1986; 1989). Antisera to LT-II toxins do not react with Type I enterotoxins and vice versa. There is 57% homology between the A subunit of Type I and II enterotoxins (Holmes *et al.,* 1990; Pickett *et al.,* 1986; 1987; 1989). In contrast , there is little homology between the B subunit of Type I and II enterotoxins (Holmes *et al.,* 1988; Pickett *et al.,* 1986; 1989). Although the B subunit of Type I and II differ in their sequences, antigenicity and

receptor binding specificity, they have similar size and overall structure, forming a pentamer to associate with the A subunit to make the holotoxin (Holmes *et al.,* 1990).

1.3.2.1 Genetics of LT toxins

LT-I produced by *E. coli* isolates from humans and animals is plasmid-encoded, but chromosomal genes may effect its level of expression (Katayama *et al.,* 1990; Neill *et al.,* 1983). Plasmids encoding LT-I usually also code for colonization factors (Echeverria *et al.,* 1986; Smith, 1984). The genes encoding LT-I have been cloned and sequenced and show high homology between LTh-I and LTp-I (Dallas *et al.,* 1979; So *et al.,* 1978; Yamamoto *et al.,* 1983; 1984). Sequences of the genes for the LT-A and -B subunits, termed *eltA* and *eltB*, revealed an overlap (Spicer *et al.,* 1981). The genes for LT subunits are transcribed as a single mRNA (Dallas *et al.,* 1979) and their pattern of codon usage, G+C content, and structural and functional similarities with CT suggest that the *E. coli* LT gene and *V. cholerae ctx* genes were derived from a common ancestor (Finkelstein *et al.,* 1987; Yamamoto *et al.,* 1987).

In contrast to CT, which is secreted into the extracellular environment, LT remains within the bacterial periplasm under experimental conditions (Hirst *et al.,* 1984). The presence of bile salts at physiological concentrations causes the release of LT into the medium, which is increased by trypsin treatment and iron starvation (Hunt and Hardy, 1991). Expression of a plasmid encoding CT in *E. coli* resulted in the holotoxin remaining localized within the *E. coli* periplasm (Hirst *et al.,* 1984; Pearson and Mekalanos 1982; Gennaro *et al.,* 1982). In contrast, expression of LT in *Vibrio* and *Aeromonas* strains led to secretion of the LT holotoxin and B subunit (Neill *et al.,* 1983; Marcello *et al.,* 1994; Michel *et al.,* 1995). These observations suggest the existence of

a specific secretion machinery which is present in *Vibrio* and closely related species. In *V. cholerae* such a set genes designated *eps* has been identified (Sandkvist *et al.*, 1993)

1.3.2.2 Structure of LT toxins

In all LT toxins, the A subunit has a molecular mass of 27 kDa and the monomeric B subunit has a molecular mass of 11.6 kDa. The total size of the holotoxin, AB_5, is 85.5 kDa. The plasmid-borne *E. coli elt-I* operon shares considerable homology with the chromosomal *ctxA,B* genes of *V. cholerae,* with 75 and 77% homology at the nucleotide level for the A and B cistrons, respectively (Mekalanos *et al.,* 1983; Dallas *et al.,* 1980). Overall the amino acid sequences are largely conserved between CT and LT-I except for the region around the cleavage site between fragment A1 and A2 (residues 192 and 193), where the homology is only about 33% between amino acids 189-212 (Dykes *et al.,* 1985a; Spicer *et al.,* 1981; 1982; Yamamoto *et al.,* 1987; Yamamoto and Yokota, 1983).

LT toxin from a porcine strain has been crystallized and the structure of the molecule studied at 2.3Å resolution (Pronk *et al.,* 1985; Sixma *et al.,* 1991; 1993). The structural model of LTp contains the B pentamer and an A subunit. LT-A has a triangular or wedge shape in one view and a V shape when rotated 90°. The A subunit is composed of an enzymatic fragment (A1) and the A2 fragment that mediates the connection of the A subunit to the B pentamer. The A1 strand contains short stretches of both α-helix and β-strands. The A2 subunit is an extended α-helix, beginning at residue 200 of the A polypeptide. The helix begins on one of the triangular sides of A1

and then continues into the middle of the central pore formed by the B pentamer (Sixma *et al.,* 1991).

Subunit A is synthesized as a single polypeptide. The CT-A subunit is nicked between residues 192 and 195 during secretion, producing 2 polypeptides, A1 and A2, linked by a disulphide bond between Cys_{187} and Cys_{199}. However LT-A remains in the periplasmic space without being nicked (Booth *et al.,* 1984; Gill *et al.,* 1979). Failure of *E. coli* to nick and secrete the toxin may explain the reduced severity of *E. coli* induced enteric disease (Pearson and Mekalanos, 1982). In both CT and LT, A1 and A2 are linked by disulphide bonds which remain unreduced, and the toxin is inactive before entering the mammalian cell.

The separation of A1 and A2 fragments by proteolytic cleavage of the main chain and reduction of the disulphide bond linking the fragments (A1 and A2), is necessary for full activity of the A subunit (Mekalanos *et al.,* 1979; Moss *et al.,* 1981; 1993). It is not completely clear why the cleavage and reduction are required. This may be needed for the separation of the A1 fragment from the rest of the toxin for efficient membrane translocation, exposure or increased accessibility of the substrate binding site or to mediate a possible conformational change in the A1 subunit (Merritt *et al.,* 1994). These authors studied the structure of the A subunit in the cleaved toxin by crystallography at 2.6 Å resolution and concluded that even though the cleavage of A1 and A2 fragments is required for biological activity of the toxin, it does not cause a conformational change by itself (Merritt *et al.,* 1994).

Structure-function studies of the A subunit by substitution of amino acids Arg_7, Glu_{110} and Glu_{112}, have shown that these residues are important for the enzymatic activity of the A subunit (Lobet *et al.,* 1991; Tsuji *et al.,* 1990).

Computer analysis of the crystallographic structure of LT-A containing single amino acids substitutions showed that the residues Leu_{41}, Ala_{45}, Val_{53}, Val_{60}, Ser_{68}, His_{70}, Val_{97} and Ser_{114} are critical in the assembly of the A subunit. Substitution of the residues Val_{53}, Ser_{63}, Val_{97}, Tyr_{104} and Ser_{114} resulted in a non-toxic molecule, which indicated the importance of these amino acids for enzymatic activity of the A subunit (Pizza *et al.,* 1994).

On the three dimensional structure of the A subunit, the NAD-binding and catalytic-sites of the A subunit are located in a cavity which consists of 18 contiguous amino acids that form an α-helix bent over a β-strand, flanked on each side by β-strands that carry the catalytic residues Glu_{112} and Arg_7 (Domenighini *et al.,* 1994; Sixma *et al.,* 1993).

The B pentamer has a ring conformation, with a hydrophobic inner surface having 25 positive and 15 negative charges lining the central pore. The B subunit monomers are tightly packed around the central pore. The B subunit monomer has two triple-stranded antiparallel β-sheets, a small N-terminal helix and a large central helix. Antiparallel β-sheets give the ring the appearance of a smooth outer surface, while long α-helixes form a helical barrel in the center, making a pore 30Å long with a diameter from 11Å near the A surface to 15Å at the other side. The pentamer has an overall diameter of approximately 64Å and a height of 40Å (Sixma *et al.,* 1991). Within the B pentamer residue Ala_{64} is in contact with Met_{31} of the next subunit around the ring and mutation of Ala_{64}→Val interferes with pentamer formation (Iido *et al.,* 1989).

A study of two-dimensional crystals of CT bound to ganglioside GM_1 showed a ring shape formed by the B pentamer lying flat on the membrane surface (Dwyer and Bloomfield 1982; Gill, 1976; Ludwig *et al.,* 1986). The A1 fragment is located away from the surface and shows minimal contact with the B pentamer. The A subunit

interacts with the B pentamer through the highly charged central pore and appears to run into the pore at its C-terminus (Sixma *et al.,* 1991; 1993).

1.3.2.3 Receptor for LT

LT toxin-membrane and toxin-receptor interactions have been studied. After oral delivery, LT-B binds to the brush border of intestinal villus epithelial cells, the cells overlying the lymphoid follicles or Peyer's patches, and undergoes transcytosis across the enterocytes via ganglioside GM_1 and galactoprotein receptors on the enterocytes (Lindner *et al.,* 1994).

Ganglioside GM_1 is a receptor for CT and LT (Cuatrecasas, 1973; Eidels *et al.,* 1983; Holmgren *et al.,* 1973; Van Heyningen, 1983). Five molecules of GM_1 on the membrane surface are bound to the B pentamer at five identical binding sites (Fishman *et al.,* 1978). The data from binding studies indicate that the oligosaccharide portion of the receptor is recognized by CT and LT. LT can interact directly with lactose and binds to the galactose portion of the sugar (Sixma *et al.,* 1992). The crystallography of an LT:galactose complex at 2.2Å resolution showed that the binding surface on the toxin contains a relatively flexible loop consisting of residues 51-60 of the B subunit. During receptor binding this loop becomes tightly ordered by forming hydrogen bonds joining the GM_1 pentasaccharide (Merritt *et al.,* 1994). LT can also interact with other receptors not recognized by CT. These include weak binding to GM_2 and to asialo-GM_1 (Fukuta *et al.,* 1988), specific binding to glycoproteins from the intestinal brush border membranes of several species (Griffiths *et al.,* 1986; Griffiths and Critchley, 1991; Holmgren *et al.,* 1982; Zemelman *et al.* , 1989) and in CaCo-2 cells (Orlandi *et al.,* 1994). Although CT recognizes the terminal sequence Gal-NAc-Gal, the terminal Gal alone is enough for LT-I binding (Fukuta *et al.,* 1988; Smith, 1984).

Binding studies have shown that Trp_{88} of the B subunit is involved in the interaction of the toxin with its receptor (Sillerud *et al.,* 1981). De Wolf *et al.* (1981) showed that this residue is in or near the receptor binding site. In the crystal structure of LT, Trp_{88} is found at the bottom of a small cavity surrounded by several loops, one of which contains Gly_{33} from the adjacent monomer. Substitution of Gly_{33} with Asp abolished the receptor binding by CT (Sixma *et al.,* 1991; Tsuji *et al.,* 1985). It has been proposed that Gly_{33} is part of a β-turn in LT and a mutation may alter the conformation of the binding site. The positions of Trp_{88} and, in the adjacent monomer, Gly_{33} suggest that the cavity is part of the ganglioside-binding site. Jobling and Holmes (1991) studied the structure and function of the B subunit of CT by substitution of several amino acids for Gly_{33}. This indicated that Gly_{33} per se is not required for the binding of CT, but a negative charge or a large hydrophobic residue interferes with binding and abolishes toxicity. Substitutions at CT-B:Trp_{88} dramatically decrease the amount of immunoreactive toxin, suggesting that Trp_{88} is crucial for the establishment or maintenance of the native conformation of the B subunit. Substitution of positively and negatively charged amino acids for Trp_{88} prevent receptor-binding activity. Substitution with neutral amino acids (Ile, Leu, Asn or Gln) or the basic amino acid His has no effect on GM_1-binding activity. These observations imply that, in this case, the chemical properties of the residues are more important than the specific amino acid to the structure and function of the protein.

1.3.2.4 Mechanism of action of LT

Binding of LT toxin to its receptor leads to translocation of the A subunit through the eukaryotic cell membrane and release of the A1 fragment into the cytosol. Two main mechanisms for membrane translocation of CT (and most likely also *E. coli*

LT) have been proposed but still need to be elaborated (Holmgren, 1994). The first involves the entry of the A1 fragment into the cytosol, either being driven into the membrane by energy released by the B subunit-GM_1 receptor binding, or entry to the cell through a disarray of the membrane lipid bilayer as a consequence, of GM_1-B-pentamer binding. In this model A1 is supposed to be located on the side of the receptor binding site of the B pentamer (Ribi *et al.,* 1988). The second mechanism implies that the B subunits unfold and extend across the membrane thereby forming a hydrophilic pore through which the A1 fragment can pass. In this model A1 is located away from the receptor binding site of the B pentamer which agrees with the CT-GM_1 crystal structure (Sixma *et al.,* 1991; 1992 and 1993).

In the eucaryotic cell, the catalytic activity of A1 fragment needs the reduction of the disulphide bond between Cys_{187} and Cys_{199} linking A1 to A2 (Gill and Rappaport, 1979; Mekalanos *et al.,* 1979; Tomasi and Montecucco, 1981). The endogenous reducing agent and mechanism of reduction are not known.

The A1 fragment hydrolyzes NAD and transfers the ADP-ribose from NAD onto the α subunit of the G_S protein of adenylate cyclase at the cytoplasmic side of the membrane. In the normal cyclase system the $G_{S\alpha}$, GTP, adenylate cyclase complex catalyzes the conversion of ADP to cAMP. When GTP is hydrolyzed to GDP by the GTPase activity of $G_{S\alpha}$ subunit, the complex dissociates and is inactivated (Gilman, 1987). ADP-ribosylation decreases the GTPase activity of $G_{S\alpha}$ and thus keeps the adenylate cyclase in an active form (Cassel and Selinger, 1977). The result of this process is the enhancement of cAMP production and accumulation in the cell, resulting in the inhibition of the absorption of sodium chloride and increased water secretion from the intestinal crypt cells, with consequent severe diarrhoea (Holmgren, 1994).

1.3.2.5 Antigenicity of LT toxins

The A and B subunits of CT and LT are not cytotoxic alone, although activated A subunit is enzymatically functional in the absence of B pentamer (Mekalanos *et al*,1979). The A and B subunits are antigenically distinct (Gilligan *et al.,* 1983) and differ in their immunogenicity. Antibodies directed against holotoxin react strongly with the B subunit and weakly or not at all with the A subunit. There are common and unique antigenic determinants among members of the serogroup I CT-LT family (Finkelstein *et al.,* 1987; Kazemi and Finkelstein, 1990). Small differences in the primary structure are sometimes associated with marked differences in neutralizing antibody specificities. Polyclonal and monoclonal antibodies against CT and LT have been produced for use in epitope mapping and binding site studies (Belisle *et al.,* 1984; Holmes and Twiddy, 1983; Kazemi and Finkelstein, 1990; 1991; Ludwig *et al.,* 1985). Mabs were used to study differences between CT-A and LT-A and some cross reactivity between CT, LTh and LTp was found (Belisle *et al.,* 1984; Finkelstein *et al.,* 1987; Holmes and Twiddy 1983; Ludwig *et al.,* 1985). It has been shown that there is extensive cross-reactivity between CT-B and LT-B with Mabs primarily recognizing the conformational epitopes of the toxins. The studies indicated the importance of several specific amino acids in antigenic determinants such as Ala_{46} and Trp_{88} of CT-B and Asn_{90} and Lys_{91} of LT-B which are involved in receptor-binding of the B subunit (Holmes and Twiddy, 1983; Kazemi and Finkelstein, 1990; Ludwig *et al.,* 1985).

1.4 Fimbriae

The ability of ETEC to bind to the epithelial surface of intestine is necessary for the establishment and colonization of bacteria in the gut and is a prerequisite for

infection. The attachment of the bacteria at the site of infection is necessary to overcome the mechanical protective factors at the epithelial surface such as peristalsis and mucosa secretion.

ETEC adhere to the specific receptor on the enterocytes of the intestinal mucosa by bacterial appendages defined as colonization factor antigens (CFA) (Levine, 1981) or putative colonization factors (PCF) (Thomas *et al.,* 1982). The colonization factor antigens of ETEC are fimbriae (pili) (Gaastra and de Graaf, 1982; Ørskov *et al.,* 1982). These are rigid, hairlike, filamentous surface appendages which are thinner than flagella (with a diameter up to about 7 nm), and are made of hundreds of copies of the structural subunits, called pilin, fimbrin or fibrillin with subunit sizes varying from 14 to 27 kDa. Some fimbriae of human ETEC have been referred to as fibrillae, because they are much thinner (about 2 nm in diameter) and more flexible.

1.4.1 *E. coli* fimbriae

The crucial stage in the pathogenesis of ETEC is the adherence of bacteria to the epithelial cells of the small intestine by their fimbriae. Several fimbrial types have been defined based on the specificity of the ligand, tissue, host, location of the fimbrial gene (on the plasmid or chromosome) and serological variation (Paranchych and Frost, 1988).

There is a relationship between the specific fimbriae and the ability of bacteria to adhere to a given host mucosal surface, due to the binding to distinct receptors on the epithelial cells (Lark *et al.,* 1986).

Many different species within the *Enterobacteriaceae* possess fimbriae, which are usually classified by their ability to agglutinate erythrocytes from different species. Based on haemagglutination (HA) reactions the fimbriae have been divided into two

groups: mannose sensitive (MSHA) and mannose resistant (MRHA) (Ottow, 1975; Pearce and Buchanan, 1980).

1.4.1.1 MSHA fimbriae

Type I or common pili are observed in different species and mediate mannose sensitive agglutination of guinea pig erythrocytes (Clegg and Gerlach, 1987; Eisenstein 1988). Type I fimbriae are rigid rods, 7nm in diameter and 1μm in length, distributed uniformly around the cell. The genes encoding type I fimbriae are located on the chromosome (Brinton *et al.,* 1961) and have been cloned and sequenced (Klemm and Christiansen, 1987). These fimbriae are not specific for ETEC and will not be discussed further.

1.4.1.2 MRHA fimbriae

Enterotoxigenic and uropathogenic *E. coli* mediate mannose-resistance haemagglutination of erythrocytes from a variety of animal species. This discussion will concentrate on mannose resistant fimbriae produced by different strains of ETEC.

The mannose-resistant haemagglutination (MRHA) is a characteristic which is used for classification of *Enterobacteriaceae* and distinguishes these colonization factors from mannose sensitive haemagglutination (MSHA), produced by type I fimbriae which are present on almost all *E. coli* strains (Ottow, 1975; Pearce and Buchanam, 1980; Ørskov and Ørskov, 1984).

1.4.2 Human ETEC colonization factor antigens (fimbriae)

The expression of colonization factor antigens (CFAs) is dependent on environmental conditions such as temperature and composition of the growth medium.

The CFAs are expressed at 37°C but not below 20°C (Gaastra and de Graaf, 1982). CFA agar has been shown to be a useful medium to provide good expression of colonization factors (Evans *et al.,* 1977). Bile salts improve the expression of a number of colonization factors (McConnell *et al.,* 1989), whereas iron and glucose repress their synthesis and the addition of glycerol or sodium acetate has no effect on expression (Evans *et al.,* 1991; Karjalainen *et al.,* 1991 a, b). Addition of exogenous leucine (100μg/ml) to the medium completely abolishes the expression of CS3 (M.B.Jalajakumari, personal communication).

1.4.2.1 Nomenclature

A number of different terms such as colonization factor antigen (CFA), coli surface antigen (CS) and putative colonization factor (PCF) have been used to describe the various fimbriae of human ETEC.

The most studied of human ETEC colonization factors are: CFA/I, CFA/II and CFA/IV (Evans *et al.,* 1975; Evans and Evans, 1978; Svennerholm *et al.,* 1988; Thomas *et al.,* 1985). CFA/I was the first colonization factor antigen described in human ETEC by Evans *et al.* (1975). It has been shown that CFA/I$^+$ ETEC strains are associated with 12 serogroups (Table 1.1) (Blancu *et al.,* 1989; Changchawalit *et al.,* 1984; Cravioto *et al.,* 1979; 1982; Drafeuille-Michaud *et al.,* 1987; Evans *et al.,* 1979; Forestuer *et al.,* 1989; Levine *et al.,* 1983; Sommerfelt, 1991). CFA/II is composed of three subcomponents termed Coli Surface associated (CS) antigens: CS1, CS2 and CS3 (Boylan *et al.,* 1988; Cravioto *et al.,* 1982; Smyth, 1982). CS1 and CS2 are fimbriae but CS3 is fibrillae (Levine *et al.,* 1984; Mullany *et al.,* 1983). All CFA/II$^+$ ETEC from 9 serogroups express CS3 either alone or in combination with CS1 or CS2 (Cravioto *et al.,* 1982; Levine *et al.,* 1983; Scotland *et al.,* 1985; Smyth, 1982; Thomas and Rowe,

Table 1.1 Properties of human ETEC fimbriae

Fimbriae	Morphology	MW kDa	Gene location	Serogroups	Reference
CFA/I	fimbriae rod	15	plasmid	O6, O7, O12, O15, O25, O32, O63, O78, O80, O85, O153	Karjalainen *et al.* (1989)
CS1	fimbriae rod	15.2	plasmid	O6, O139	Perez-Casal *et al.* (1990)
CS2	fimbriae rod	15.4	chromosome	O6	Froehlich *et al.* (1995)
CS3, CS3a	fibrillar flexible	15.11& 15.24	plasmid	O8, O78, O80, O85, O115, O139, O154, O168	Jalajakumari *et al.* (1989) Cassels and Wolf (1995)
CFA/III	fimbriae rod	25.3	plasmid	O25	Taniguchi *et al.* (1995)
CS4	fimbriae rod	14.9	plasmid	O25	Cassels and Wolf (1995)
CS5	fimbriae flexible	18.6	plasmid	O6, O29, O92, O114, O128	Manning *et al.* (1987)
CS6	?	15, 15.8	plasmid	O27, O92, O148, O153	Cassels and Wolf (1995)
CS7	fibrillar, helical	21.5		O15, O78, O103, O114, O127, O128	McConnell (1991)
CS17	fimbrial rod	17.5	plasmid	O8, O12, O15, O48, O114,O128, O146	McConnell *et al* (1990)
PCFO9(CS13)	fibrillar flexible	27		O9, O143	Heuzenroeder *et al.*(1990) McConnell (1991)
PCFO20	fimbrial rod	25	chromosome	O20	Viboud *et al.* (1993)
PCFO148	fimbrial, curly	unknown		O148	Knutton *et al.* (1987)
PCFO159	fimbrial rod	18.6	plasmid	O159	Tacket *et al.* (1987)
PCFO166	fimbrial rod	15,5 & 17	plasmid	O166	McConnell *et al* (1991)
2230	undetermined	16	plasmid	O25	Darfeuille-Mauchaud *et al.* (1986)
8786	undetermined	15.34	plasmid	O117	Aubel *et al.* (1991)
Longus	fimbrial rod	22	plasmid	O2, O6, O8, O20, O49, O128, O139, O148	Girón *et al.* (1994)

1982; Thomas and Rowe, 1982). The expression of CS1 and CS2 is dependent upon the biotype of the strains. Strains of biotype A express CS1 and biotypes B, C and F produce CS2 (Cravioto *et al.,* 1982; Smyth, 1982).

CFA/III was identified in ETEC strains from Japan (Honda *et al.,* 1984). The strain was O25:H$^-$ which also expressed CS6, but CFA/III positive strains of serotype O25:H16 from other geographical areas have also been isolated (Honda *et al.,* 1984; McConnell and Rowe, 1989). The role of CFA/III in colonization of bacteria in the rabbit intestine in RITARD model was shown later by Svennerholm *et al.* (1992).

CFA/IV, initially termed PCF8775, has been confirmed to be a colonization factor (Svennerholm *et al.,* 1988b). Like CFA/II, CFA/IV consists of three subcomponents: CS4, CS5 and CS6 (Thomas *et al.,* 1985). CS6 is expressed alone or in combination with CS4 or CS5. The CS4 and CS5 are fimbrial in nature whereas CS6 is non-fimbrial.

Several other less well characterized fimbriae have been identified on human ETEC which have been designated as putative colonization factors (PCF). These include PCFO9 (Heuzenroeder *et al.,* 1990), PCFO148 (Knutton *et al.,* 1987), PCFO159 (Tacket *et al.,* 1987), PCFO166 (McConnell *et al.,* 1989c), PCFO20 (Viboud *et al.,* 1993). Other fimbrial types include CS7 (Hibberd *et al.,* 1990), CS17 (McConnell *et al.,* 1990) and CS19 (Grewal *et al.,* manuscript in preparation). Some other putative adhesins have been defined simply as antigen 2230 (Forestier *et al.,* 1987) and antigen 8786 (Aubel *et al.,* 1991).

Svennerholm *et al.* (1992) indicated that PCFO159, CS7 and CS17 were colonization factors using a non-ligated intestine model (RITARD).

CS7 was identified on an ETEC strain of serotype O15:H11 which, like CS5, agglutinated bovine, human and guinea pig red blood cells. These CS factors have other

similarities such as the size of the major subunit and the helical structure of the fimbriae. There are some common epitopes between CS5 and CS7 which are not exposed on the intact fimbriae but cross-react in the denatured form. CS7 was able to adhere to human enterocytes and a CS7-expressing strain colonized rabbit intestine in the RITARD model (Hibberd *et al.,* 1990; 1991).

CS17 are rigid fimbriae with a diameter of ~7.5 nm identified on ST^- LT^+ strains of O8:H9, O15:H^-, O48:H26, O114:H21 and O146:H19 (Deneke *et al.,* 1981; McConnell *et al.,* 1989b; 1990). CS17 is encoded by a 100 MDa plasmid which also codes for the toxins. CS17 positive strains exhibit a 17 kDa protein which reacts with antisera against the CS4 fimbrial subunit, similar to CFA/I, CS1 and CS2. No cross-reaction is observed with the native fimbrial subunit, suggesting the existence of common epitopes among these five fimbrial subunits which are not exposed on the native fimbriae (Hibberd *et al.,* 1991).

PCFO9 has been identified from ST^- LT^+ strains isolated from Australia (Heuzenroeder *et al.,* 1990) and Peru (McConnell, 1991). The adhesin antigen has fibrillar structure, similar to CS3, as shown by electron microscopy, with a subunit size of 27 kDa.

A putative colonization factor called PCFO20 was identified on a ST^+ LT^+ ETEC strain of serotype O20:K27:H^-, isolated from a child with diarrhoea in Argentina and termed PCFO20 (Viboud *et al.,* 1993). This strain adhered to the CaCo-2 cell line and produces a 27 kDa protein at 37°C which reacts with antisera against purified fimbriae in immunoblot analysis. There was no cross-reactivity between this PCF and other known CFAs or PCFs.

PCFO148 are curly fibrillae with a diameter of 3nm that are expressed by human ETEC of serotype O148:H28 (Knutton *et al.,* 1987). These adhere to human enterocytes suggesting a role as a potential colonization factor antigen.

PCFO159 has been found in serogroup O159:H20. The fimbriae are composed of a 19 kDa subunit protein which is encoded by a plasmid that also encodes ST and LT (McConnell and Rowe, 1989; Tacket *et al.,* 1987).

PCFO166 is morphologically similar to CFA/I but antigenically different from other fimbriae in human ETEC. PCFO166 is able to agglutinate human or bovine red blood cells in the presence of mannose (MRHA). The fimbriae are encoded by a plasmid of 98 MDa. Immunoprecipitation and immunodiffusion studies of this PCF suggested that the fimbriae may be composed of two subunits of different molecular mass (McConnell *et al.,* 1989b). The N-terminal sequence of both polypeptides (the first 25 amino acids) are identical and are similar to the N-termini of CFA/I, CS1, CS2, and CS4 suggesting that the two proteins are modified forms of the same gene product and belong to the same fimbrial class as these proteins (Sommerfelt *et al.,* 1992).

Non-fimbrial antigen 2230 has been demonstrated on the ETEC strains of serotype O25:H16. The major subunit has a size of 16 kDa and does not mediate haemagglutination. A 66 MDa plasmid encodes the adhesin antigen (Forestier *et al.,* 1987).

Antigen 8786 is a non-fimbrial adhesin antigen which shows MRHA with human and bovine red blood cells. It was demonstrated on an O117:H4 strain that produces only ST. This antigen was shown to adhere to brush borders of human enterocytes and to CaCo-2 cells. The protein has a size of 16.3 kDa and is antigenically distinct from previously known CFAs and PCFs of human ETEC. The gene encoding antigen 8786 is located on a 70 MDa plasmid (Aubel *et al.,* 1991).

Girón *et al.* (1994) demonstrated a new pilus on human ETEC strain E9034 of serotype O8:H9 and designated Longus. Longus was also found in a considerable number of serotypes (Table 1.1) (Girón *et al.,* 1995a). These pili form bundles which are 20μm long, composed of a repeating subunit of 22kDa found at one pole of the cell. They are expressed under anaerobic conditions on blood agar. There is similarity between the N-terminal amino acid sequence of this pilin and the toxin co-regulated pilus of *Vibrio cholerae*, CFA/III, the bundle-forming pilus of enteropathogenic *E. coli* and to a lesser extent with type IV pili. Therefore this pilus is placed in class B of type IV pili (Girón *et al.,* 1994).

1.4.2.2 Antigenic properties of CFAs

The primary structures of the major subunits of most of the CFAs and PCFs have been identified (Clark *et al.* 1992; Clark and Manning, unpublished results; Hamers *et al.,* 1989; Jalajakumari *et al.,* 1989; Jordi *et al.,* 1991; Karjalainen *et al.,* 1989; Prez-casal *et al.,* 1990; Wolf *et al.,* 1989) and the antigenic determinants of some of them have been studied.

Monoclonal antibodies against various colonization factors have been produced (Honda *et al.,* 1989; Lopez-Vidal and Svennerholm, 1990; Lopez-Vidal *et al.,* 1988; Viboud *et al.,* 1993; Worobec *et al.,* 1983) and used for characterization, epitope mapping and epidemiological studies. Potential antigenic determinants of the CFA/I major subunit have also been suggested based on predictions of the secondary structure of the protein (Klemm and Mikkelsen, 1982) and two epitopes predicted within the N-terminal 46 amino acids. Lopez-Vidal *et al.* (1988) characterized 25 CFA/I agglutinating Mabs and compared one of them with a non-agglutinating Mab. Both Mabs reacted with isolated fimbriae and with the 46 N-terminal amino acid peptide. The non-agglutinating

Mab could not recognize CFA/I on the whole bacteria but the agglutinating Mab did, suggesting two different epitopes for the two Mabs on CFA/I, one of which is not exposed in native form in CFA/I.

Some cross-reactivity has been observed among different colonization factors. One Mab against CFA/I gave cross-reactivity with K99 fimbriae of animal ETEC in ELISA but not in Western blot analysis. CFA/I, CS1, CS2, CS4 and PCFO166 are antigenically distinct in ELISA and immunodiffusion studies (McConnell *et al.,* 1989b; 1990) but CFA/I, CS1 and CS2 cross-react with polyclonal antisera against CS4 in immunoblot analysis (McConnell *et al.,* 1989b). Antisera against CS4 also react with the CS17 fimbrial subunit. Antisera against CFA/I has weak reactivity with CS1, CS2 and CS4 and similarly CFA/I, CS2 and CS4 reacted weakly with an antibody to CS1.

The specificity of two Mabs for CS3 was studied and found that both Mabs reacted with CS3 from different strains in denatured form. However, one of them could not recognize native CS3 on the bacteria expressing both CS3 and CS2, suggesting different epitopes for these Mabs (Lopez-Vidal and Svennerholm, 1990).

Antigenic studies of colonization factors using polyclonal and monoclonal antibodies suggest that there are common structural epitopes (Worobec *et al.,* 1983) and some shared epitopes which are not surface exposed and therefore can not be recognized in the native form on the whole cells, but give cross-reactivity in immunoblot analysis.

1.4.2.3 Receptors for ETEC fimbriae

ETEC producing different colonization factors have been shown to attach to erythrocytes (Evans *et al.,* 1977), buccal cells (Walser *et al.,* 1992), human enterocytes (Knutton *et al.,* 1985; Viboud *et al.,* 1995) and brush border membrane of rabbit enterocytes (Wenneras *et al.,* 1990). These studies have been used to characterize the

receptors for colonization factors. Different cell lines including Hela cells (Guth *et al.,* 1995), HT29 (Neeser *et al.,* 1989) and Caco-2 (Darfeuille-Michaud *et al.,* 1990; Viboud *et al.,* 1995) have also been used to investigate the binding of ETEC. Darfeulle-Michaud *et al.* (1990) studied the adherence of the ETEC expressing CFA/I and CFA/II, and demonstrated that these colonization factors mediate the binding of the ETEC to Caco-2 and human enterocytes. They concluded that these cells have similar receptors. Binding of different colonization factors to these cells has also been studied. Strains producing CFA/I, CS2, CS4+CS6, CS5+CS6, CS7, CFA/III+CS6 and PCFO166 bind to both Caco-2 and human enterocytes, suggesting the presence of similar binding sites for these colonization factors on both cells. Bacteria expressing CS3 or PCFO9 bound well to human enterocytes but not to Caco-2 cells, suggesting different receptors in these cells (Viboud *et al.,* 1995).

It has been shown that cells expressing 8786 antigen adheres to the Caco-2 cells and the binding is not inhibited by purified CFA/I, CFA/II, CS6 and 2230 antigens, suggesting different receptors for these fimbriae on these cells (Darfeuille-Michaud *et al.,* 1990). Bacteria expressing CS6 only are able to colonize the rabbit intestine in the RITARD model (Svennerholm *et al.,* 1988) but do not adhere to human small intestinal enterocytes (Knutton *et al.,* 1989). Other colonization factors such as CFA/I, CS1, CS2, CS3, CS4, CS5 and PCFO159 mediate adherence of the bacteria to human enterocytes (Hinson *et al.,* 1987; Knutton *et al.,* 1987).

Almost all human ETEC show mannose resistant haemagglutination (MRHA) of erythrocytes from various species. It has been shown that CFA/I binds to sialic acid containing ganglioside GM_2 and sialoglycoproteins of erythrocytes (Bartus *et al.,* 1985; Faris *et al.,* 1980; Naaser *et al.,* 1988). A sialoglycoprotein, with a size of 26 kDa was isolated from human erythrocyte membranes, and was able to bind to CFA/I expressing

bacteria (Pieroni *et al.,* 1988). It has been demonstrated that the subcomponents of CFA/II (CS1, CS2 and CS3) and CS4 of CFA/IV apparently bind to asialo-ganglioside GM_1 (Sporsem Oro *et al.,* 1990). The binding of purified CFA/I and CFA/II to cell membrane components of the human intestinal cell line HT29 and rabbit intestinal brush borders has been studied and it was shown that CFA/I bound to a similar membrane component with a size of 30-35 kDa from both cell type. CFA/II^+ ETEC also bound these molecules and CS3 bound strongly to two components of rabbit intestinal brush border membrane with a size of 120-140 kDa. This was absent in various cultured cell lines. Two non-intestinal cell lines tested did not contain the components for binding CFA/I and CFA/II.

Extraction of lipids from the cell membrane with chloroform-methanol did not affect the binding of CFA/I and CFA/II, suggesting that the receptor is a (glyco)protein. Treatment of the membrane with neuraminidase abolished the binding of fimbriae indicating that sialic acid may be part of the receptor. The 120-140 kDa CS3 receptors from rabbit intestinal brush borders were not affected by neuraminidase treatment (Wenneras *et al.,* 1990) but proteinase K treatment abolished the binding, confirming the proteinaceous nature of the CS3 receptor. Oxidation of the intestinal cell membrane with sodium meta-periodate abolished CS3 binding, indicating the binding of CS3 to carbohydrate moieties of glycoproteins. Inhibition experiments showed that GM_1, asialo-GM_1 and GM_2 inhibited the binding of the CS3, suggesting the carbohydrate sequence, GalNAcβ1-4Gal, is the CS3 binding moiety of the glycoprotein. Electron microscopy showing the localization of this disaccharide around CS3- positive bacteria supports these findings (Wenneras *et al.,* 1995).

1.4.2.4 Genetics and biogenesis of CFAs

The genes encoding most of the human ETEC fimbriae are located on plasmids which usually also encode ST and/or LT. The genes encoding colonization factors are organized in clusters which include a periplasmic chaperone, usher (high molecular weight outer membrane protein) and the structural gene encoding the major subunit. Table 1.1 shows properties of the human ETEC fimbriae which will be discussed in the following sections.

1.4.2.4.1 CFA/I

CFA/I is encoded on plasmids with sizes of 89-108 kb. Two regions (region 1, 7.7 kb and region 2, 2.1 kb), separated by ~40 kb, are required for the expression of CFA/I (Smith *et al.,* 1982). These regions have been cloned (Willshaw *et al.,* 1983), and the nucleotide sequences determined (Hamer *et al.,* 1989; Jordi *et al.,* 1992; Karjalainen *et al.,* 1989; Savelkoul *et al.,* 1990). Region 1 consists of *cfaB* (the structural subunit gene), *cfaA* which encodes a protein homologous with CooB (see section 1.4.2.4.2.1) and probably needed for assembly of fimbriae, and *cfaC* and *cfaE* encoding proteins of unknown functions (Jordi *et al.,*1993).

Region 2 contains one gene designated *cfaD* or *cfaR* (Jordi *et al.,* 1993) which encodes a 265 amino acid protein (CfaD) corresponding to a positive regulator (Grewal *et al.,* 1993; Savelkoul *et al.,* 1990). The protein has been suggested to be a DNA-binding protein related to the AraC family of transcriptional activators and is homologous to *Rns* (regulation of CS1 and CS2 antigens of CFA/II, Caron *et al.,* 1989).

The %G+C content of *cfaA*, *cfaB*, *cfaC*, *cfaE* (each between 30-40%) and *cfaD* (28%) are low for *E. coli* genes (50%) (Jordi *et al.,* 1992) and thus have been suggested to have originated in other bacterial species.

1.4.2.4.2 CFA/II

CFA/II is composed of three subcomponents, CS1, CS2 and CS3 with subunit sizes of 16.3, 15.3 and 14.8 kDa, respectively (Smyth, 1982). The expression of the particular CS fimbrial phenotype is dependent on the biotype of the host strain (Cravioto *et al.,* 1982; Smyth, 1982). With CS1 being expressed in biotype A whereas biotypes B, C and F produce CS2 fimbriae. All biotypes express CS3 (Scotland *et al.,* 1977).

Expression of plasmids encoding CS1 and CS2 from serotype O6:H15:K16 or H^- in other O serotypes or in *E. coli* K-12 resulted in the production of CS3 only. The reverse transformation of plasmids from these CS3-producing strains into strains of serotype O6:H15:K16 or H^- lacking the CS fimbriae associated plasmid resulted in expression of CS1 and CS2 fimbriae (Smyth, 1986). It has been suggested that chromosomal determinants found only in strains of serotype O6:K15:H16 or H^- are required for the expression of CS1 or CS2 (Twohig *et al.,* 1988) and that both chromosomal and plasmid information is needed for the expression of CS1 and CS2 (Smyth, 1986). Caron *et al.* (1989) demonstrated the existence of a plasmid encoding a positive regulatory gene (*rns*) required for the expression of CS1 and CS2. Genes for *rns* and *cfaD* are highly homologous but, unlike *cfaD* which is located on the same plasmid as the structural genes, *rns* is located on a separate plasmid (Perez-Casal *et al.,* 1990; Willshaw *et al.,* 1990b).

1.4.2.4.2.1 CS1

The gene encoding the major subunit of CS1, designated *cooA* (for **co**li surface antigen **o**ne), was cloned from a different plasmid to that encoding *rns*. The deduced size of CooA is 15.2 kDa, very close to that observed by Western blot analysis using a CS1-specific antiserum (16 kDa). There is 92% similarity and 55% identity between CooA and the major subunit of the CFA/I, CfaB , at the amino acid sequence level (Rerez-Casal *et al.,* 1990). The product of the first gene in the *coo* locus, CooB, has an apparent size of 26 kDa, and is involved in the assembly of CooA (Scott *et al.,* 1992).

1.4.2.4.2.2 CS2

Perez-Casal *et al.* (1990) suggested the chromosomal location of the CS2 genes, which like CS1, CFA/I and CS4 are positively regulated by plasmid located *rns*. A DNA fragment of 5.7 kb required for production of CS2 in *E. coli* K12 has been cloned and sequenced (Froehlich *et al.,* 1995). This fragment contains four open reading frames, *cotB, cotA, cotC* and *cotD* of which *cotA* encodes the major pilus subunit. These genes show homology with those involved in expression of CS1 and CFA/I. CotA and CotB proteins interact with the CooC and CooD proteins of CS1 to form pili, and the CooA and CooB from CS1 interact with CotC and CotD to produce pili.

1.4.2.4.2.3 CS3

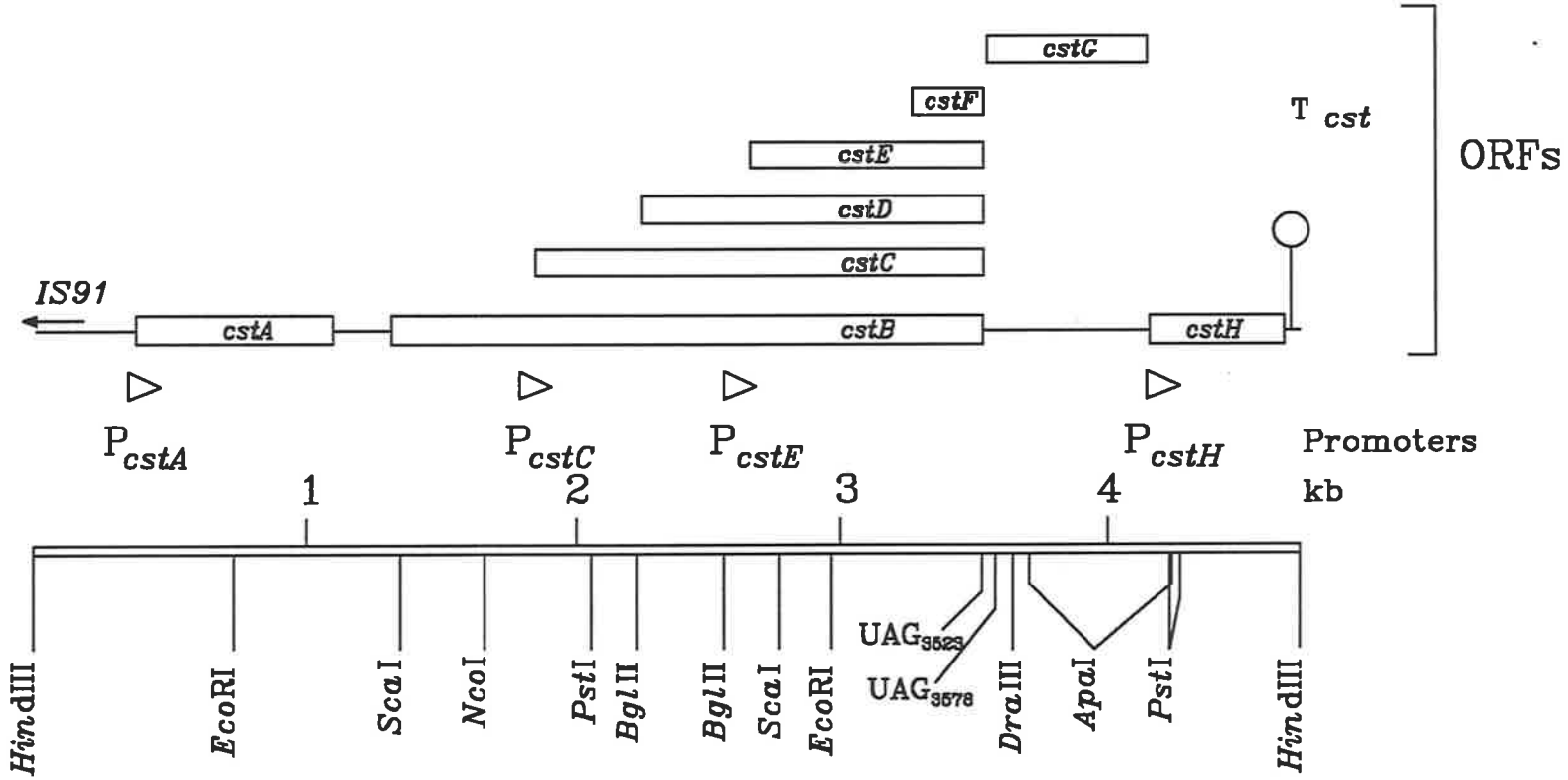
The molecular cloning of the genetic determinants encoding CS3 fimbriae has been achieved independently by three groups (Boylan *et al.,* 1987; Manning *et al.,* 1985; Willshaw *et al.,* 1988). The nucleotide sequence of the minimal coding region for the biogenesis of CS3 has been reported (Jalajakumari *et al.,* 1989) and the presumptive

genes designated nnkDa, where nn is a number approximating the predicted size (x 1000) of the polypeptide it encodes, and the potential promoters as Pnn. In order to adopt a convenient terminology by which the CS3 biosynthesis genes are referred to in future, they were renamed as *cstA* to *cstH*, where *cst* stands for ***c***oli ***s***urface antigen ***t***hree (Fig 1.3). The most striking feature of the CS3 gene cluster is that a number of the genes completely overlap. Four genes (*cstA, cstB, cstG* and *cstH*) are placed contiguously, but not overlapping with each other, however another four ORFs (*cstC, cstD, cstE* and *cstF*), are completely contained within *cstB*. The UAG triplet at nt 3523 is needed for the termination of some of the polypeptides (*cstB, cstC, cstD, cstE* and *cstF*) (Jalajakumari and Manning, manuscript in preparation). A very intriguing feature is the presence of an inframe UAG triplet (UAG_{3578}) within one of the genes, *cstG*. Suppression, or some other form of bypassing of this codon, appears to be an integral part of CS3 regulation (Jalajakumari *et al.,* 1989).

Cell fractionation experiments and studies of the homology to other known ETEC pilus systems helped to assign some structural roles to the genes in the CS3 operon (Jalajakumari and Manning, manuscript in preparation). Thus, CstA, which is present in the periplasmic fraction and shows homology to a number of chaperone proteins, is probably needed for the transport of pilin to the outer membrane. The CstE may be associated with CstA (periplasmic chaperone) and CstH (pilin subunit), both of which show homology to FaeE, FanE and PapD in the K88, K99 and Pap systems, and to FimG (type 1) and PapE (Pap system). The similarity of CstG to PapH (Båga *et al.,* 1987) suggests that this protein may form the basal part of the pilus itself and helps in the cell anchorage of the pilus structure. The CstB protein shows homology to PapC of the Pap system (Norgren *et al.,* 1987), FaeD in the K88 system (Mooi *et al.,* 1983), FanD in K99 (Roosendaal and De Graaf, 1989) and FimD in type I pili systems (Klemm and

Fig 1.3 Genetic organization of the *cst* operon

The thin line represents the region DNA with relevant restriction sites. Open boxes show ORFs. Promoters are indicated by triangles. Terminator to *cstH* is also shown. *cstH* represents the major pili subunit gene.


cstG
cstF
cstE
cstD
cstC
T cst
ORFs
IS91
cstA
cstB
cstH
P cstA
P cstC
P cstE
P cstH
Promoters
1
2
3
4
kb
HindIII
EcoRI
ScaI
NcoI
PstI
BglII
BglII
ScaI
EcoRI
UAG3523
UAG3578
DraIII
ApaI
PstI
HindIII

Christiansen, 1990). These proteins correspond to the ushers which form channels in the outer membrane through which pilins (eg CstH) are transported and assembled into the pilus structure. Other proteins (CstC, CstD and CstF) may have structural roles, in conjunction with CstB, in forming the channel. Studies are underway to identify the roles of these proteins in CS3 biogenesis.

1.4.2.4.3 CFA/III

In strains producing either LT or CS6, CFA/III is encoded by a plasmid of 86-90 kb, whereas it is encoded by a separate plasmid of 52 kb in strains producing only CFA/III (McConnell and Rowe, 1989). A 17.4 kb DNA fragment containing the gene necessary for expression of CFA/III has been cloned from a 55 kb plasmid of *E. coli* strain 260-1 (Shinagawa *et al.,* 1993), and the structural gene, *cofA,* with a size of 714 bp encoding a 238-amino acid protein was sequenced. CofA is a precursor of CFA/III pilin which, after cleavage between Gly_{30} and Met_{31} forms the mature pilin. The N-terminal 30- amino acid sequence of mature CFA/III has high homology with TcpA pilin of *Vibrio cholerae* and thus it has been located in type IV class B pili (Taniguchi *et al.,* 1995).

1.4.2.4.4 CFA/IV or PCF8775

CFA/IV is composed of three subcomponents, CS4, CS5 and CS6. The genes involved in biogenesis of CFA/IV are plasmid-located and the strains may contain several plasmids.

O25:H42 ETEC strains producing CS4+CS6 hybridize with the regulatory gene of CFA/I, *cfaD*. It has also been shown that *cfaD* regulates the expression of CS4 but not of CS6. However, the DNA fragment hybridizing to *cfaD* is located on the same plasmid that also encodes the CS6 antigen (Willshaw *et al.,* 1990). The regulatory gene for the expression of CS4 has been cloned on a 3.3 kb *Hin*dIII fragment and had identical restriction pattern as *cfaD*. This regulator also promotes the expression of CS1, CS2 and CFA/I fimbriae (Willshaw, 1991). The structural genes for CS4 expression are located on a second plasmid, and a further plasmid encodes enterotoxin. Enterotoxin production is therefore not linked to either the regulatory genes or the genes encoding CS antigens. In most strains CS5 and CS6 are coded for by the same plasmid. No DNA fragment encoding CS4 has yet been cloned.

The genes encoding CS5 antigens have been cloned as a 8.5 kb DNA fragment. The minimal coding region necessary for the production of CS5 is about 7 kb (Heuzenroeder *et al.,* 1989). The nucleotide sequence of the DNA fragment encoding the major subunit has been determined (Clark *et al.,* 1992). The subunit is synthesized as a precursor of 20.8 kDa (203 amino acids) containing a typical signal sequence, with a mature protein of 181 amino acids corresponding to a size of 18.6 kDa. The amino acid sequence of the subunit is different from subunits of other colonization factors but shows some homology to the subunit of porcine ETEC F41 (Clark *et al.,* 1992).

Regulatory genes from strains of serogroup O115 and O167:H5 producing CS5 have been cloned (Willshaw *et al.,* 1991). The products of the genes from O167 were similar to *cfaD*, *rns* and the CS4 regulatory genes, and mediate the expression of CFA/I, CS1, CS2 and CS4 antigens. There is 87% homology between this gene and *cfaD* at the nucleotide level (Dehaan *et al.,* 1991).

Two variants of CS6 antigen with sizes of 14.5 and 16 kDa have been identified (McConnell *et al.,* 1988; Wolf *et al.,* 1989). A 3 kb DNA fragment from O167 and O27 strains has been cloned and expressed in *E. coli* K-12, and three signal sequence containing proteins with sizes of 15.6, 16.7 and 26 kDa were recognized. Antisera against CS6 reacted with the 15.6 and 16.7 kDa proteins. It was not clear whether the two proteins represented two types of antigens present in CS6 fimbriae or were processed from a common precursor (Willshaw *et al.,* 1988). The cloned DNA fragment obtained from a strain of serogroup O167 encoding CS6 has been sequenced. Three open reading frames encoding proteins of 140, 160 and 220 amino acids were determined which had sizes slightly smaller than those observed by Willshaw *et al.* (1988). All three proteins had signal sequences and the smallest one was considered to be the CS6 subunit.

1.4.2.4.5 PCFO9

The genes determining the biosynthesis of PCFO9 from an ETEC strain of O9:H$^-$ have been cloned (Heuzenroeder *et al.,* 1990). The region encoding the major pilin subunit (27 kDa) was localized using a synthetic oligonucleotide probe derived from N-terminal amino acid sequence of the subunit. All genes involved in the biosynthesis of PCFO9 have been identified and sequenced. The amino acid sequences of products differ from those of other human ETEC fimbriae but have high homology with those associated with the synthesis of K88 fimbriae from porcine ETEC (Clark and Manning, manuscript in preparation).

1.4.2.4.6 PCFO20

The genes involved in biogenesis of the PCFO20, a newly identified human colonization factor, are located on the chromosome (Viboud, 1995). The structural gene of PCFO20 was cloned as a 3.3 kb DNA fragment and expressed in *E. coli* K-12. Sequencing of 1.3 kb of this fragment revealed an ORF, *fotA,* whose product, FotA, had a similar molecular mass as that of the PCFO20 fimbrial subunit. *fotA* encodes a 20.5 kDa protein with a 21 amino acid signal sequence. The mature protein has a size of 18.1 kDa and is homologous to the subunit of porcine 987P (82% similarity at amino acid level) but not to human ETEC (Viboud *et al.,* 1993; Viboud, 1995).

1.4.2.4.7 Longus

The structural gene for longus pili, *lngA,* which is located on a large plasmid has been cloned on a 15 kb fragment and expressed in *E. coli* K-12 (Girón *et al.,* 1994). It has been shown that the *lngA* gene has high association with CFA/II, CFA/I and CFA/IV producing ETEC strains. *lngA* is also found in a considerable number of ETEC serotypes and is more associated with ST^+ ETEC than with LT^+ST^+ or LT^+ ETEC strains (Girón *et al.,* 1995a).

The genetic organization of other human ETEC colonization factors has not yet been extensively studied.

1.5 Expression of heterologous peptides on the bacterial surface

One of the most interesting developments in molecular biology has been the construction of new proteins with novel properties. By using recombinant DNA technology one can modify the amino acid sequence of a protein by substitution, deletion or insertion of amino acid residues, or by fusing two or more proteins to form a hybrid protein.

The insertion of foreign peptides into cell surface exposed proteins (referred to as vector proteins) and expression of the hybrid proteins on the surface of a bacterium such as *E. coli* can be used to study the topology and structure of the molecule, as well as the interactions between the bacterium and its environment (including its potential hosts). This approach has a potential application in vaccine development and diagnosis, and the search for active peptides for therapeutic use and immunopurification.

Foreign antigenic determinants from viral envelope proteins (Klemm and Hedgaard, 1990) or domains of bacterial proteins such as cholera toxin (Newton *et al.,* 1989) have been expressed at the cell surface. The size of the insert varies from a few amino acids to over 60 residues. With the C-termini of the vector proteins possibly being able to accept larger insertions.

The stability and function of hybrid protein depends on the nature of the insert and, more importantly, on the site of insertion (Bouges-Boquet *et al.,* 1984). It is possible to insert a variety of peptides of limited size in most cell surface sites of the vector, but the composition of the insert should not be extremely hydrophobic or charged (Agterberg *et al.,* 1990a; Charbit *et al.,* 1988).

Permissive sites for insertion of foreign peptides within a vector may be determined experimentally. If the gene encoding a protein has been cloned, but nothing is known about the sequence and structure of the protein, it may be possible to recognize permissive sites by random insertion of the foreign peptide and then the evaluation of the hybrid proteins. If the sequence and/or structure of a protein is known, it may be possible to predict permissive sites within the vector. Hydrophilic sequences or turn regions and loops that are located on the surface of the molecule (or cell surface) are good candidates as insertion sites of foreign peptides. The regions of a protein which show variation between species are potentially the most likely to be best for mutagenesis and insertion. If the C-terminus of a protein is exposed, it could also be a suitable site for introduction of a foreign peptide.

Two classes of cell surface-associated proteins have been used as vectors for expression of heterologous peptides on the surface of bacteria. These are outer membrane proteins and subunits of cell appendages (flagella and fimbriae).

1.5.1 Outer membrane proteins

Several outer membrane proteins including LamB, PhoE, OmpA, and TraT have been used for insertion of foreign epitopes to study their topology and application in vaccine development.

1.5.1.1 LamB

LamB is a trimeric integral outer membrane protein of *E. coli* that mediates the entry of maltose and maltodextrins into the cell (Nikaido and Vaara, 1985). LamB is also a cell surface receptor for bacteriophage λ (Charbit and Hoffnung, 1985). Based on the deduced sequence of the protein and knowledge of residues affecting phage receptor activity, a model for folding of the protein in the outer membrane was suggested (Charbit *et al.,* 1984). Insertion of foreign epitopes into three sites within the LamB protein have been used to test this model.

LamB was the first vector developed for cell surface expression of foreign epitopes. The C3 epitope from poliovirus (residues 93-103 of the VP1 protein of the virus) was inserted after residues 153, 253 and 374. The epitope was expressed on the cell surface as shown by immunoelectron microscopy of intact cells using a Mab against C3. Insertion did not have any effect on the structure and function of the LamB protein and supported the proposed model for folding (Charbit *et al.,* 1986a,b; 1991). Several epitopes with different sequences and sizes of up to about 60 amino acids, have been inserted into the site adjacent residue 153. They were expressed on the cell surface without disruption of the structure and function of the LamB molecule (Charbit *et al*, 1988). Insertion of longer peptides abolished the function of LamB (Charbit *et al*, 1988; Reeves *et al.,* 1990). Several other foreign antigenic determinants, including portions of PreS2 region of hepatitis B virus (Charbit *et al.,* 1987), epitopes of the major outer membrane protein of *Chlamydia trachomatis* (Hayes *et al.,* 1991) and a peptide from interferon-γ (IFN-γ) (Gargiulo *et al.,* 1993) have been used for insertion and expression in LamB. Immunization of animals with hybrid proteins results in production of antibodies against both the carrier and inserts. Recombinant Chlamydial-LamB hybrid

proteins have also been expressed in an *aroA* mutant of *Salmonella typhimurium* and shown to be immunogenic in mice (Hayes *et al.,* 1991).

1.5.1.2 PhoE

The PhoE protein from *E. coli* has been used for expression of antigenic determinants on the bacterial surface. PhoE also forms a trimer in the outer membrane, protein providing a transmembrane channel to allow the entry of small hydrophobic molecules (Nikaido and Vaara, 1985). In the proposed model for folding of PhoE, the protein traverses the outer membrane 16 times in a β-sheet structure with eight exposed regions (loops) at the cell surface (Vander Ley *et al.,* 1986; Vander Ley and Tommassen, 1987). These loops were considered as suitable sites for the insertion and expression of foreign epitopes. An antigenic peptide of the structural protein VP1 of the Foot-and-Mouth disease virus (FMDV) was inserted in the second, fourth and eighth exposed loops. The expression of the VP1 antigenic determinant on the surface of the cell was demonstrated by ELISA, using intact cells and a Mab against the foreign antigen. The second and eighth loops both tolerated insertion of about 50 and 30 amino acid residues, respectively, and the vector could accept two insertions simultaneously (Agterberg *et al.,* 1987; 1990a). Immunization of mice with partially purified hybrid proteins, having different antigenic determinants of the VP1 protein, resulted in the production of antibody to the peptides (Agterberg *et al.,* 1990b).

This approach has also been used to study the topology of the PhoE protein. By insertion of an antigenic determinant of VP1 protein of FMDV Struyve *et al.* (1993) demonstrated that the third loop of PhoE is located within the pore channel. B-cell

epitopes of the VP1 protein of FMDV were inserted into the exposed region of PhoE. Immunization of guinea pigs with hybrid proteins elicited protective antibodies against the virus and the animals were protected against viral challenge (Agterberg *et al.,* 1991; Tommassen *et al.,* 1993).

PhoE has been used for the expression of a T-cell epitope (amino acids 180-188) of the 65 kDa heat shock protein of *Mycobacterium tuberculosis*. The hybrid protein induced proliferation of epitope-specific T-cell clones *in vitro* and induced an *in vivo* T cell response against the 180-188 T cell epitope. The results indicate that the peptide is immunogenic and antigenic in hybrid PhoE protein (Agterberg *et al.,* 1991; Hogervorst *et al.,* 1990; Janssen *et al.,* 1994; Tommassen *et al.,* 1993).

1.5.1.3 OmpA

Outer membrane protein OmpA has also been used for expression of heterologous peptides. OmpA is a major structural component of the membrane of Gram negative bacteria with a monomeric structure. The protein traverses the outer membrane with eight β-sheet segments forming four surface exposed regions (Vogel and Jahnig, 1986) which are receptor binding sites for various phage and colicins (Lugtenberg and Van Alphen, 1983; Manning and Reeves, 1976; Manning *et al.,* 1976; Morona *et al.,* 1985). The third domain of the molecule differs in sequence between *E. coli* and *Shigella dysenteriae* (Braun and Cole, 1982), and has been used for insertion and expression of various fragments containing antigenic epitopes of VP1 protein of FMDV. Expression of the heterologous epitopes on the bacterial surface was demonstrated by indirect immunofluorescence and immunogold labelling. Hybrid

proteins with large insertion stimulated production of anti-virus antibody in rabbits (Ruppert *et al.,* 1994).

1.5.1.4 TraT

TraT is an oligomeric outer membrane lipoprotein encoded on plasmids of the IncF group, that has been used for insertion and expression of foreign antigenic determinants. TraT mediates resistance to the bacteriocidal activity of serum and reduces the ability of the bacteria to act as recipients in conjugation with bacteria harbouring closely related plasmids (surface exclusion) (Sukupolvi and o'Connor, 1990).

The C3 epitope of poliovirus was inserted at five sites (at residues 61, 125, 180, 200 and 216) within the TraT protein. Three hybrid proteins with insertions at residues 61, 200 and 216 were shown to express the foreign epitope on the cell surface as indicated by immunoassay using an anti-C3 Mab (Harrison *et al.,* 1990; Taylor *et al.,* 1990).

1.5.2 Cell appendages

Cell appendages such as flagella and fimbriae (pili) are composed of hundreds of subunits of a monomer, flagellin and fimbrial (fimbrin, fibrillin or pilin) subunits, respectively. These subunits have variable and exposed domains which have been considered as good candidates for the insertion and expression of heterologous antigenic determinants.

1.5.2.1 Flagellin

The bacterial flagellum, which mediates motility, comprises a basal structure that serves to anchor it on the membrane, and a long external shaft. This shaft is a polymer of a single protein, flagellin. Flagellin is an approximately 500 amino acid protein with a central hypervariable domain that can be replaced by foreign peptides.

An 11 residue peptide from hen egg-white lysozyme has been expressed in the flagellin from *E. coli* K-12 (Kuwajima *et al.,* 1988). The flagellin from *Salmonella* has been used for insertion of various epitopes. Newton *et al.* (1989) inserted an epitope of cholera toxin B subunit (residues 50-64) into a *Salmonella* flagellin. The hybrid flagellin acted normally with respect to motility and the epitope was expressed at the flagellar surface. Immunization of mice with an *aroA* flagellin-negative strain of *S. dublin* expressing the hybrid flagella elicited antibody responses to cholera toxin.

Oligonucleotides encoding epitopes of S (residues 122-137) and pre-S2 (residues 120-145) domains of hepatitis B virus surface antigen were inserted into the hypervariable region of a *Salmonella* flagellin gene. A flagellin negative *aroA* mutant of *S. dublin* expressed the chimeric flagella as shown by immunoblotting using antibodies against both flagellin and hepatitis B virus surface antigen. Animals immunized with live recombinant bacteria developed antibodies against hepatitis B virus (Wu *et al.,* 1989).

The insertion of a peptide sequence from the surface antigen Pre-S1 (residues 12-47) of hepatitis B virus into *Salmonella* flagellin resulted in assembly of functional flagella which were immunogenic. Antibodies directed against the viral antigen developed when the bacteria were injected intraperitoneally (i.p) into mice (Xiao-Song He *et al.,* 1994).

Intraperitoneal immunization of animals with live *Salmonella* expressing the 15 N-terminal amino acids of the M protein of *Streptococcus pyogenes* type 5 within flagella on the cell surface, induced the production of antibodies against M protein. Following immunization, mice were partially protected against a lethal challenge of *S. pyogenes* type 5 (Newton *et al.,* 1991; Stocker, 1990).

A B-cell epitope (residues 201-224) of Murray Valley encephalitis virus has also been inserted into the flagellin of *Salmonella*. Expression of the epitope on the cell surface along the flagellum was demonstrated by immunoblot and immunogold electron microscopy using a Mab specific to the epitope (Whittle *et al.,* 1994).

1.5.2.2 Fimbriae

Fimbriae are long, flexible, filaments composed of hundreds of identical major subunits as well as minor components which enable the bacteria to adhere to various surfaces. Sequence comparison of related fimbriae reveals conserved structural motifs and implies the presence of variable domains which usually correspond to the major antigenic determinants. Different fimbriae have been used for insertion and expression of heterologous epitopes.

1.5.2.2.1 Type 1 fimbriae

These fimbriae have been used for the insertion of two epitopes of hepatitis B surface antigen (residues 122-137 and 140-146), the C3 epitope from the VP1 capsid protein of type 1 polio virus and an epitope of the VP1 capsid protein of FMDV into the

fimbrial subunit. The results indicated that epitopes up to 18 residues could be expressed at two sites (after residues 28 and 57) without any effect on the overall structure of the fimbriae, and were detectable on the surface of the bacteria (Hedegaard and Klemm, 1989; Klemm and Hedegaard 1990).

1.5.2.2.2 P-fimbriae

P-fimbriae consist mainly of a major subunit with some minor components comprising the actual adhesin at their tip. The major fimbrial subunit determines the antigenic properties of the fimbriae and has been used for classification of P-fimbriae into serotypes F7-F13 (Ørskov and Ørskov,. 1985). There are five hypervariable segments (HRs 1-5) containing the major antigenic epitopes of the P-fimbriae (Van Die *et al.,* 1987; 1988). The HRs of the major subunit of F11 fimbriae appeared to be suitable for insertion of foreign antigenic determinants.

Replacement of HR4 with an epitope of FMDV had no effect on assembly of the hybrid fimbriae and the foreign epitope could be recognized using a Mab to FMDV (Van Die *et al.,* 1988). It has also been demonstrated that HR1 and HR4 can accept foreign epitopes of up to 14 amino acids from different sources. The chimeric fimbriae could be assembled and exposed the inserted peptides. Hybrid fimbriae containing an epitope of FMDV induced the production of antibodies to the insert when injected into mice (Van Die *et al.*, 1990). Recently P-fimbriae have been used to express Gonadotropin releasing hormone (GnRH), which regulates reproduction in mammals. GnRH was inserted into the hypervariable region of F11 fimbriae. The hybrid fimbriae were expressed on the bacterial surface. Immunization of female rats and bull calves with purified hybrid

GnHR-fimbriae affected the reproductive characteristics of animals (Van der Zee *et al.,* 1995).

1.5.2.2.3 K88 fimbriae

K88 fimbriae, produced by enterotoxigenic *E. coli* in domestic animals, mediate attachment of bacteria to the intestinal epithelium and facilitate colonization of the gut (Klemm, 1985). There are three distinct serotypes of K88 -K88ab, K88ac and K88ad - with common antigenic determinants (a) and variable antigenic determinants (b, c and d) (Dykes *et al.,* 1985b). The variable domains of K88ac (residues 164-171) and K88ad (residues 208-221) were substituted by a linker sequence, and the hormone somatostatin and two epitopes of the influenza virus haemagglutinin were cloned into these linkers. Of the two recombinant pili containing influenza epitopes, one stimulated the production of antibodies in rat against the insert. Hybrid pili expressing somatostatin were able to be recognized by both monoclonal and polyclonal antibodies (Thiry *et al.,* 1989). Replacement of the variable region (residues 163-173) of K88 with different epitopes did not affect the biogenesis of pili and the epitopes were detected on the pili. Immunization with hybrid fimbriae induced antibodies against foreign antigenic determinants (Bakker *et al.,* 1990).

1.5.2.2.4 CS31A

CS31A is a plasmid-encoded fibrillar antigen which is found in bovine and human enterotoxigenic or septicaemic *E. coli* and produces a capsule-like structure around the cell. The CS31A antigen is related to K88 fimbriae with 46% homology at the amino acid level (Girardeau *et al.,* 1988; 1991). There are two variable regions in the subunit, V2 (residues 123-150) and V3 (residues 186-221) and the only continuous epitope of the

native protein is located in V3. These domains have been used for insertion and replacement with different foreign epitopes. The results showed that replacement of the V3 domain with heterologous viral epitopes can be performed without a drastic effect on CS31A biogenesis.

Foreign epitope expression on the bacterial cell surface and immunization of mice with these bacteria resulted in the production of antibodies against the foreign epitope (Bousquet *et al.,* 1994). Fusion of heterologous peptides (with different physical properties) of up to 19 amino acids to the N-terminus of the CS31A subunit was also performed. The hybrid antigen was cell-surface exposed as indicated with immunogold electron microscopy (Der Vartanian *et al.,* 1994).

1.6 Vaccines against human ETEC

Diarrhoeal diseases are an important world wide problem, particularly in developing countries and in travellers to these areas. In developed countries, children also suffer from acute diarrhoea often leading to hospitalization and sometimes death (Black, 1993; Savarino and Bourgeois, 1993). Diarrhoeal diseases are estimated to cause 3.7-4.6 million deaths in infants and children each year (Black, 1993). ETEC has been identified as an important causative agent in diarrhoea, resulting in 400-650 million cases and 500,000-800,000 deaths annually in children under five years old (Kaper and Levine, 1988; Reid *et al.,* 1993; Black, 1986). Two virulence factors, colonization factor antigens (pili or fimbriae) and enterotoxins (ST and/or LT) are involved in the adhesion of ETEC to the intestinal brush border and subsequent disease. Although diarrhoeal diseases can be effectively treated by oral rehydration therapy, prevention of the illness is preferred.

Investigations have demonstrated that toxins and adhesins of bacteria could be useful vaccine antigens against ETEC (Evans *et al.,* 1988). Numerous studies have been undertaken to develop vaccines based on these components, but as yet no vaccine is in current human use.

1.6.1 Purified fimbrial vaccines

Fimbriae are good immunogens and experimental and natural infection by ETEC elicits the production of antibodies against pili and other components of the bacteria (Levine *et al.,* 1984; Rivas *et al.,* 1995). Thus it has been proposed that a purified fimbrial vaccine consisting of different CFAs could serve as an effective vaccine. Fimbrial vaccines based on K88, K99 and 987P antigens are routinely used in farm animals to prevent ETEC infections (Klemm, 1985; Moon and Bunn, 1993).

A purified preparation of type 1 pili was the first vaccine evaluated in humans. It was hoped that this vaccine would provide broad protection against *E. coli* strains expressing different CFAs and enterotoxins because of the production of type 1 pili by pathogenic and non-pathogenic *E. coli.* Parental administration of the vaccine stimulated the production of sIgA anti-type1 pili antibodies but did not protect vaccinees against ETEC challange (Levine *et al.,* 1984).

Several attempts have been made to develop a fimbrial vaccine in animals and humans. Oral immunization with a purified CFA/II (CS1+CS3) vaccine led to rises in antibodies to CFA/II in only two out of ten volunteers (Levine *et al.,* 1986) and did not give significant protection against homologous pathogenic ETEC challenge. Duodenal administration of the vaccine via an intestinal tube resulted in the production of significant specific sIgA response in four out of five volunteers. These results imply the adverse effects of stomach acid and susceptibility of fimbriae to proteolytic digestion

(Schmidt *et al.,* 1985). To overcome these problems in fimbrial vaccines, a biodegradable polymer (Lactide-co-glycolid) (PLG) which has been used for the delivery of other protein antigens (Eldridge *et al.,* 1989; Moldoveanu *et al.,* 1989) was tested. Purified CFA/I antigen was encapsulated in biodegradable PLG polymer microspheres and used for a single dose immunization of rabbits via an intragastric tube. The immunization induced a strong systemic antibody response of the IgG subclass to CFA/I. One out of three rabbits elicited CFA/I sIgA antibodies (Edelman *et al.,* 1993).

PLG has also been used for intraduodenal immunization of rabbits with CFA/II (CS1+CS3). The results showed that the vaccine was safe and immunogenic and stimulated the production of IgG antibodies to CFA/II. Following vaccination, Peyer's patch cells could be shown to respond *in vitro* to CFA/II. Anti-CFA/II antibody secreting B cells were also found in the spleens (Reid *et al.,* 1993). The immunogenicity and protective capacity of purified CFA/II antigen encapsulated in PLG was evaluated in human volunteers immunized four times weekly via intestinal tubes (Tacket *et al.,* 1994). Five out of ten volunteers developed IgA CFA/II antibody-secreting cells (ASC). Five out of ten had sIgA anti-CFA/II in jejunal fluid. 30% of the vaccinees were protected in a challenge with the heterologous ETEC strain 8 weeks after the first immunization. Two of the protected vaccinees had the highest numbers of ASC and highest titers of sIgA suggesting that this strategy is promising. However, additional studies need to be undertaken to develop more effective systems for delivery of purified fimbrial vaccines and to reduce the number of vaccine doses required.

1.6.2 Toxoid vaccines

Three antigens of ETEC are able to stimulate the production of antibodies and mediate protection against ETEC: LPS, fimbriae and enterotoxin (LT) (Ahren and

Svennerholm, 1982; Evans *et al.,* 1988; Levine, 1990). Immunization with LPS and specific fimbriae do not protect against heterologous strains. Immunization with toxins which are common to all strains of ETEC elicit the production of antibodies which may protect against homologous and heterologous strains (Klipstein *et al.,* 1981a; b; c).

ST is a low molecular weight (18-19 amino acids), non-immunogenic peptide toxin which can become immunogenic when coupled to a large molecular weight carrier. Several different carrier molecules have been used to prepare immunogenic toxoids of STa including: outer membrane protein OmpC (Saarilahti *et al.,* 1989), Bovine serum albumin (Frantz and Robertson., 1981), LT-A (Sanchez *et al.,* 1986), LT-B (Clements, 1988; 1990) and IgG binding fragment of *Staphylococcus aureus* proteinase A (Lowenadler *et al.,* 1991).

Native and synthetic STa have been used to construct fusion proteins with different carriers through chemical procedures and recombinant DNA technology (Clements, 1990; Houghten *et al.,* 1985; Klipstein *et al.,* 1984; Sanchez *et al.,* 1988). These fusion proteins have been used to elicit antibody responses and promising results were recently obtained using LT-B::ST fusions which mediated mucosal protection against both ST and LT toxins following oral immunization (Cardenas and Clements, 1993).

Synthetic modified ST-peptides corresponding to the 15 C-terminal amino acid residues of STa have been made. Peptides in which one or two cysteines were replaced with alanine were non toxic but reacted with an ST neutralizing Mab (Svennerholm *et al.,* 1988). Fusion of a decapeptide homologous to STa to cholera toxin B subunit resulted in a hybrid protein able to react with STa antibodies and to give rise to STa antibodies with weak neutralizing activity (Sanchez *et al.,* 1988).

LT is a high molecular-weight, highly immunogenic protein which is immunologically and physicochemically related to cholera toxin. The immunogenicity of LT and CT and their binding B subunit, have been well-studied (Gilligan, 1983; Pierce *et al.,* 1988).

LT-B has been used to construct fusion proteins with ST, and immunization experiments demonstrated the production of antibodies to both ST and LT-B (Clements, 1990; Cardenas and Clements, 1993; Aitken and Hirst, 1993). A non-toxic LT lacking ADP-ribosylating activity could stimulate the production of LT antibodies in mice (Douce *et al.,* 1995). Recently Haq *et al.* (1995) expressed the LT-B gene in transgenic plants and demonstrated that immunization of mice with a crude soluble extract from leaves induced serum and mucosal anti-LT-B immunoglobulins with neutralizing activity in cell protection assays. Feeding mice with fresh transgenic potato tubers also induced immunity (Haq *et al.,* 1995).

There has been interest in the development of toxoid vaccines based on epitopes of CT-B which are conserved in LT-B. It has been shown that antibodies directed against a peptide vaccine corresponding to a region of CT-B which is conserved in LT-B can react and neutralize LT-B biological activity (Ghose and Karush, 1988; Jacob *et al.,* 1984; 1986). Klipstein *et al* (1986) have constructed a non-toxic synthetic peptide consisting of the 18 amino acids of ST and a 26 amino acid peptide of LT-B. Oral immunization of volunteers resulted in serum and intestinal antibody rises against both components (Klipstein *et al.,* 1986). It has also been demonstrated that antibodies against the B subunit of CT gives protection against LT producing ETEC (Clements *et al.,* 1986; 1988).

1.6.3 Whole cell vaccines

An alternative vaccine approach for the protection against ETEC is to use killed ETEC bacteria alone or in combination with the B-subunit of the LT. Clemens *et al.* (1988) have demonstrated that the combined cholera toxin B subunit/killed whole cell oral cholera vaccine conferred short-term cross-protection against LT-producing ETEC among rural Bangladeshi children and women in field trials. The same vaccine gave a similar protection against diarrhoea associated with LT^+ ETEC in travellers to Morocco (Peltola *et al.,* 1991) which implied cross protection was mediated by CT-B. Colicin E2-killed ETEC (ST^+, LT^+, O78:H11, CFA/I) have been used for immunization of young healthy volunteers, and this demonstrated the induction of intestinal IgA and serum IgG antibodies against CFA/I and LT, indicating that colicin E2-treated bacteria can deliver antigens to the gut immune system. This study also showed partial protection of vaccinees against ST^+ LT^+ CFA-homologous and heterologous ETEC (Evans *et al.,* 1988). Similarly, a formalin-killed vaccine derived from ETEC producing CFA/I and CFA/II and CT-B subunit was safe, and gave rise to specific intestinal IgA (CFA/I 82%, CFA/II 82% and CT-B 91%) and also weaker serum IgA and IgG antibodies in most of a group of volunteers immunized orally (Ahren *et al.,* 1993).

1.6.4 Live oral vaccines

An effective immunization against ETEC has to be able to elicit protective immunity at the level of the mucosal surface of the small intestine, the site of action of ETEC. Mucosal antibodies inhibit adherence of the bacteria to the mucosal surfaces. Induction of mucosal antibodies can be achieved by the delivery of the antigen at the mucosal surfaces by the oral administration of attenuated strains or avirulent derivatives of *Salmonella* spp. as carriers of the antigen.

1.6.5 Attenuated *E. coli* vaccines

It has been demonstrated that infection with an ETEC strain can produce a 100% diarrhoeal attack rate and that protection to challenge developed in seven out of eight volunteers (Levine *et al.*, 1979). Thus, it was inferred that an attenuated strain of *E. coli* producing the important antigenic determinants had the potential to induce strong, protective immunity. Experimental studies showed that ingestion of a single dose of 10^9, 10^{10} or 6×10^{10} cells of CFA/II-positive (CS1+CS3) O6:H16 strain (a spontaneous derivative of a wild type ETEC lacking *est* and *eltA,B* genes) resulted in significant rises in intestinal sIgA antibodies to CFA/II in 11 out of 14 volunteers. All volunteers had positive stool cultures, most had positive duodenal fluid cultures and 2 out of 19 had mild, transient diarrhoea. Challenge with a different O:H serotype ETEC (LT^+ and ST^+) with the same CFA/II antigen caused diarrhoea in only 3 out of 12 vaccinees. The bacterium was isolated from five of six controls (7×10^3 cells/ml) but from only one of 12 vaccinees (10^1 cells/ ml) (Levine *et al.*, 1986) implying a dramatic effect on colonization. These results demonstrated that an attenuated live vaccine could induce the production of sIgA which inhibited the ability of challenge bacteria from colonizing the small intestine, and was more effective than a purified fimbrial vaccine. Although this live attenuated vaccine resulted in significant protection, the mild diarrhoea in about 15% of volunteers demands the need for research to develop an efficient immunogenic live oral vaccine without side effects.

1.6.6 Live oral *Salmonella* vaccines

Another attractive alternative approach for the delivery of antigens to the intestinal immune system, and the induction of a protective immune response, is the use of attenuated *Salmonella spp.* expressing heterologous antigens. Attenuated *Salmonella* provide a prolonged high level production of the antigens with a fate as the antigen in a natural infection. The antigen is also presented directly into the gut-associated lymphoid tissue and thought to cause maximal stimulation for the production of sIgA (Cardenas and Clements, 1992). This system has been used for the expression of fimbriae of animal ETEC. K88 and K99 fimbriae have been expressed in an attenuated strain of *Salmonella typhimurium*, *galE* mutant G30 (Attridge *et al.,* 1988; Morona *et al.,* 1994; Stevenson and Manning, 1985). Oral and intraperitoneal immunization of mice with *S. typhimurium* G30 expressing K88 induced the production of anti-K88 antibodies in both the mice serum and gut (Stevenson and Manning, 1985) and *S. typhimurium* G30 expressing K88 and K99 have been administered orally to adult pigs and elicited significant serum (Morona *et al.,* 1994), colostral and milk antibodies to the fimbrial antigens (Attridge *et al.,* 1988). The response was sufficient to give positive protection in suckling piglets (Attridge *et al.,* 1988).

Mutant *Salmonella spp.* vaccine strains have been used for the expression of the heat-labile toxin B subunit. Plasmids encoding LT-B were introduced into *Salmonella typhi* (*galE* mutant) and *Salmonella enteritidis,* and immunization of mice with these strains elicited the production of serum and mucosal antibodies (Clements and El-Morshidy, 1984; Clements *et al.,* 1986). Mice immunized orally with a *S. typhimurium aroA* mutant expressing LT-B developed neutralizing antibodies to the antigen (Dougan *et al.,* 1987). The gene encoding LT-B was also integrated into the chromosome of a *Salmonella* mutant and the expression and stability of the foreign peptide in the carrier strains demonstrated (Clements and Cardenas, 1990). Cardenas and Clements (1993)

constructed a fusion protein composed of LT-B and ST with an eight amino acid proline-containing linker in between. The hybrid protein was expressed in an *aroA* mutant of *S. dublin* and used for the oral immunization of mice, which then developed serum IgG and mucosal IgA that could recognize LT-B and neutralize native ST in the suckling mouse assay.

The genes encoding human ETEC fimbriae (CFA/I and CFA/II) have been introduced into *Salmonella typhi* vaccine strain, CVD908, on a plasmid (Giron *et al.,* 1995; Xu *et al.,* 1993) and integrated into the chromosome (Xu *et al.,* 1993). In all cases the fimbriae were expressed on the surface of the vaccine strain.

Based on our knowledge of the factors involved in the pathogenesis of ETEC and their role in immune mechanisms against ETEC infection, it seems that an effective vaccine must contain the protective antigens of ETEC and be given orally to elicit antibodies to both colonization factors and toxins. Thus the ideal vaccine should be composed of a combination of different colonization factor- and toxin-antigens. This vaccine could be made of different killed or attenuated live bacterial strains expressing different colonization factors and toxins (such as LT-B). Attenuated *Salmonella spp* expressing these antigens can be used as live vaccines as they present a high level of antigen directly into the immune system similar to the natural infection.

An alternative way to make the appropriate combination is to construct, through recombinant DNA technology, strains which can express different antigens simultaneously. Giron *et al* (1995) introduced two plasmids encoding CFA/I and CS3 fimbriae into a *Salmonella typhi* vaccine strain CVD908 and *E. coli* K-12 and demonstrated the simultaneous expression of both fimbriae on the surface of the bacteria. This provides promise for the co-expression of different antigenic determinants that are

never seen together in nature and may be useful in vaccine development against diarrhoea.

1.7 Aims of this study

In recent years, numerous attempts have been made to develop a vaccine against human ETEC based on colonization factor antigens (fimbriae) or toxoids, but as yet no vaccine is currently in use. An ideal vaccine candidate should contain a combination of fimbrial- and toxoid-antigens that could be given orally.

Relevant to this, an interesting area of progress in molecular biology has been the construction and expression of fusion proteins on the surface of carrier strains, with potential application in vaccine development.

The overall aim of this study is to develop a potential candidate vaccine strain against ETEC, based on CS3 pili.

The study was designed initially to investigate antigenic variation and epitope analysis of the CS3 subcomponent of CFA/II$^+$ enterotoxigenic *E. coli* with the aim of predicting permissive site(s) for insertion of foreign epitopes within the major subunit gene, *cstH*. The approaches to be taken involved the production of a panel of Mabs against CstH, cloning, sequencing and immunological analysis of CS3 from different ETEC strains, constructing C- and N-terminal deletions and fusions of CstH and analysis of their reactivity with the Mabs.

Second approach was the development of CS3 pili (fimbriae) as a delivery system for the expression of foreign antigenic determinants, by the insertion of foreign epitopes into different regions within the major structural subunit, CstH. The foreign epitopes chosen were an epitope of LT-B and mature ST.

These various hybrid proteins and fimbriae could then be evaluated for the immunogenicity, and as potential candidate vaccine.

Chapter 2

Materials and Methods

2.1 Growth media

Nutrient broth (NB) composed of Lab Lemco (Oxoid, 10 g/1), Bacto peptone (Oxoid, 5 g/l) and NaCl (5 g/l), was the general growth medium for bacterial cultivation. Luria broth (Miller, 1972) composed of Bacto tryptone (Difco, 10 g/l), Yeast extract (Difco, 5 g/l) and NaCl (5 g/1). The ψB medium which was used for competent cell preparation, comprised of Bacto yeast extract (5 g/l), Bacto tryptone (20 g/l), $MgSO_4$ (5 g/l) pH adjusted to 7.6 with KOH. Wild type, mutants and hybrid CS3s were expressed on CFA medium which consisted of casamino acids (Difco 10g/l), Bacto yeast extract (Difco, 1.5 g/l), $MgSO_4.3H_2O$ (0.05 g/l), and $MnCl_2.4H_2O$ (0.005 g/l).

Solidified media were prepared by the addition of BBL technical grade Agar or Bacto Agar (Difco, 15 g/l) to broth medium. Antibiotics were added to broth and solid media at the following final concentrations: ampicillin (Ap), 50-200 μg/ml; kanamycin (Km), 50 μg/ml; rifampicin (Rif), 200 μg/ml; chloramphenicol (Cm), 25 μg/ml; tetracycline (Tc) 8 μg/ml.

RPMI medium 1640 for tissue culture was obtained from Gibco laboratories.

Incubations were at 37°C unless otherwise specified. Liquid cultures were grown in 20 ml McCartney bottles.

5-Bromo-4-Chloro-3-Indolyl Phosphate (X-pho) and 5-Bromo-4-chloro-3-indolyl-β D-Galactopyranoside (X-gal) were dissolved in dimethyl formamide at a final concentration of 20mg/ml, and 1 ml of this solution was added to 500 ml of Nutrient agar (NA; final concentration of 40 μg/ml) to make X-pho and X-gal plates, respectively Isopropyl-β-D thiogalactopyranoside (IPTG) was dissolved in Milli-Q (MQ) water and filter sterilised to give a concentration of 24 mg/ml, and 1 ml of this solution was added to 500 ml of NA to make IPTG plates (final concentration of 48 μg/ml).

2.2 Chemicals and reagents

All chemicals used were of analytical grade. Phenol, polyethylene glycol 8000 (PEG) ethanol, methanol, propan-2-ol, iso-amyl-alcohol, hydrochloric acid, glycerol, sodium chloride, ammonium acetate, sucrose and sodium dodecyl sulphate (SDS) were from BDH chemicals. Tris base (Tris), glycine and the four deoxyribonucleotide triphosphates (dATP, dCTP, dGTP and dTTP) were purchased from Boehringer Mannheim. 3-[N-morpholino] propanesulphonic acid (MOPS), ethylene-diamine-tetra-acetic acid (EDTA), Triton X-100, calcium chloride, magnesium sulphate, magnesium chloride, potassium chloride, potassium-di-hydrogen orthophosphate, sodium citrate, sodium hydrogen carbonate, sodium hydroxide, ammonium chloride, chloroform and formaldehyde were from Ajax chemicals. Mineral oil was Primol 352 (Esso). Adenosine-5`-triphosphate (ATP), l,4-dithiothreitol (DTT), Isopropyl-β-D thiogalactopyranoside (IPTG), 5-bromo-4-chloro-3-indolyl-β D-galactopyranoside (X-gal), 5-bromo-4-chloro-3-indolyl phosphate (X-pho), herring sperm DNA, Tween 20, cobalt chloride, bovine serum albumin (fraction V), hypoxanthine, aminopterin, thymidine, L-glutamine, dimethyl sulfoxide, Anti-rabbit and Anti-mouse Ig FITC

conjugate were from Sigma. Foetal bovine serum was from Cytosystem, Australia. PEG 4000 was from Gibco laboratories.

Milli-Q water was purified using the Milli Q water purification system (Millipore Corp.) with a measured resistance to conductivity of 18 MΩ/cm.

Antibiotics were purchased from Sigma (Ap, Gm, Km, Rif, Strep) or Calbiochem (Cm, Tc). All other antimicrobial agents (dyes, detergents and antibiotics) were purchased from Sigma Chemical Co., BDH Chemicals Ltd., Glaxo, or Calbiochem.

Electrophoresis grade reagents were: acrylamide and ultra pure N, N' methylene bis acrylamide and urea (BRL); ammonium persulphate and agarose (Biorad), and N,N,N,N-tetramethyl-ethylenedia mine (TEMED) (Sigma).

2.3 Enzymes

Ribonuclease A (RNase) and lysozyme were obtained from Sigma. Glycogen and Proteinase K were from Boehringer Mannheim.

Restriction endonucleases were purchased from Amersham, Boehringer Mannheim or New England Biolabs, and used according to the supplier's instructions.

Other DNA modifying enzymes were purchased from New England Biolabs (Vent DNA polymerase, T4 DNA ligase), Boehringer Mannheim (calf intestinal phosphatase, terminal transferase) and Progen (T4 DNA ligase).

Sequencing kits, using dye-labelled terminators, and dye-labelled primers were purchased from Applied Biosystems. The Erase-a-base kit used to produce nested deletions of plasmid DNA was purchased from Promega.

2.4 Bacterial strains and plasmids

The Enterotoxigenic *E. coli* strains and *E. coli* K-12 strains used for transformation of plasmids and expression of the proteins are showed in Table 2.1. *Salmonella* strains used for expression of hybrid CS3 pili and immunization are listed in Table 2.2. Plasmid vectors and plasmids which were used in this study are listed in Table 2.3.

2.5 Maintenance of bacterial strains

Strains in routine use were stored as a suspension of freshly grown bacteria in glycerol (32% v/v) and peptone (0.6% w/v) at -70°C. All strains were subcultured from single colonies before storage. A loopful of the glycerol suspension was streaked onto an NA plate (with appropriate antibiotic or without antibiotic) and incubated overnight to provide fresh culture.

Strains were maintained as lyophilized cultures for long term storage. To prepare lyophilized cultures, bacteria from fresh cultures were suspended in a small volume of sterile skimmed milk and 0.2 ml of suspension were dispensed into sterile freeze drying ampoules. The end of the ampoule was plugged with cotton wool. The samples were lyophilized in a freeze drier. The ampoules were sealed and stored at 4°C. When required, an ampoule was opened and its contents resuspended in a small volume of NB and used to inoculate 10 ml NB and grown with shaking at the appropriate temperature, streaked onto nutrient agar plates and incubated overnight. Single colonies from the plates were selected for subsequent use or storage.

Table 2.1 *Escherichia coli* strains used in this study

Strain	Phenotype/Genotype	Source/Reference
ETEC strains		
PB176	E13734/78 O6:H16 ST+ LT+ CFA/II+	B. Rowe
PB176p-	E13734/78 O6:H16 ST- LT- CFA/II-	B. Rowe
E41	LT+ CFA/II+	Honda *et al.* (1983)
E9034	O8:H9 ST+ LT+ CFA/II+ (CS3)	Scotland *et al.* (1985)
E1392-75	O6:K15:H16 ST+ LT+ CFA/II+ (CS3+CS1)	Lopez-Vidal and Svennerholm (1990)
E247425-1	O6:K15 ST+ LT+ CFA/II+ (CS3+CS17)	Svennerholm, A-M
E248750-1	O6:K-:H16 ST+ LT+ CFA/II+ (CS3+CS2)	Lopez-Vidal and Svennerholm (1990)
Escherichia coli K-12 strains		
DH5α	*F-, ϕ80dlacZΔM15, Δ(lacZYA-argF)U169. deoR, recA1, endA1, hsdR17(rk-, mk+), supE44, thi1, gyrA96, relA1,* λ^-	Bethesda Research Laboratories, USA
MZ1	*his ilv rpsL* $galK_{am}$ *pglΔ8 (bio-uvrB)ΔH1*	Zuber *et al.* (1987)
E219	*MZ1 htrA63::mini Tn10*	Chris Clark
E4109	*F'traD36,* $proAB^+$*, lacIqZΔM15 rpsL, thr, leu, thi, lac7 galK, galT, ara, tonA, tsx, dam, dcm, supE44, Δ(*lac^- *proAB)*	Marinus, MG
C75a	HfrC, *tonA22. pho64*[a]*, ompF627(*$T2^R$*), relA1, pit10 spoT1,* λ^-	B. Bachmann (A. Garen, Unpublished)
G206	HfrC, *tonA22. phoB62, ompF627(*$T2^R$*), relA1, pit10 spoT1,* λ^-	B Bachmann (Kreutzer *et al.*, 1975)

Table 2.2 Salmonella strains used in this study

Strain	Genotype/Phenotype	Refrence
LB5010	*metA22 metE551 trpD2 ilv452 hsdLT6 hsdSA29 strA120 galE*	Stevenson and Manning (1985)
P9108 (G30)	*galE F- lam-*	Stevenson and Manning (1985)

Table 2.3 Plasmids and cloning vectors

Plasmid	Markers	Reference
pBR322	Ap^R, Tc^R	Bolivar et al (1977)
pPM484	Ap^R	Manning et al (1985)
PACYC184	Cm^R, Tc^R	Chang and Cohen (1978)
pGEM3Zf+	Ap^R	Promega
pGEM7Zf+	Ap^R	Promega
pGP1-2	Km^R	Tabor and Richardson (1985)
pBC KS+	Cm^R	Stratagene
pPM3500	Ap^R	C. A. Clark
pBluescript (S/K)	Ap^R	Stratagene

2.6 Synthesis of oligodeoxynucleotides

Oligodeoxynucleotides (oligos) described in Table 2.4 were synthesised on an Applied Biosystems model 381A DNA synthesiser, using chemicals purchased from Applied Biosystems or Ajax Chemicals (acetonitrile). Oligos were purified after synthesis using oligo purification cartridges purchased from Applied Biosystems according to manufacturer's specification and then purified by butanol extraction.

2.7 Animals

For the preparation of immune-spleen cells female BALB/C mice were used for immunization using purified CS3 or *Salmonella typhimurium* G30 strain carrying plasmids encoding hybrid CS3. Adult New Zealand white outbred rabbits were used for raising antibodies against CS3.

2.8 Transformation of *E.coli*

2.8.1 Preparation of competent cells

Competent cells for use in transformation were made ~~either~~ by a modified method of Brown *et al.* (1979). In the first method, *E. coli.* K-12 strains were grown on fresh ψA at 37°C overnight. A 5 ml ψB culture was inoculated with a single colony and grown overnight. This was diluted 1:20 into 100 ml ψB and incubated with shaking further to an optical density (OD) 650 of 0.6. Cells were chilled on ice for 10 min, pelleted at 4°C in a bench centrifuge, resuspended in 40 ml ice cold 30 mM potassium acetate, pH 5.8, 100 mM $RbCl_2$, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% (v/v) glycerol and incubated on ice for 5 min. Cells were then pelleted in a bench centrifuge at 4°C and resuspended in 4 ml of ice-cold 10 mM MOPS pH 6.5, 10 mM $RbCl_2$, 75 mM $CaCl_2$,

Table 2.4 Oligonucleotides used in this study

Name/number	sequence	Usage
M13-Rev	CAGGAAACAGCTATGACC	Sequencing
M13-20 primer	GTAAAACGACGGCCAGT	Sequencing
#565	ACGCAGAGCGGCAGTCTTG	Sequencing of *cstH::phoA*
#299	AGTGGTCCTGAAAGCATGAATAGT	DNA hybridization with *est* gene
#302	TATCTGACCGAGACCAAAATTGT	DNA hybridization with *eltB* gene
#735	CGTTACAGACATCATCAG	Sequencing of *est* gene
#736	CTTGCTATTACCACTGCC	Sequencing of *est* gene
#738	GTCCTGAAAGTCTAGATAGTAGCA	Amplification and mutagenesis
#739	TTTATATTATCTAGATAGCACCCG	of the mature *est* gene
#741	TTTCTGATACTCTAGAGAATGGTAC	Making *Xba*I site in *cstH*
#742	GTTGGATAAAAACCTAGGAAATGA	Making *Sty*I site in *cstH*
#743	GTTAAAACTACCCTAGGGAGTCAT	Making *Sty*I site in *cstH*
#782	AAACCAGAACGATTGCGC	Sequencing the DNA fragment
#783	ATCGATACCGTTCAGACG	containing *est* gene (pPM4565)
#794	GTTCCAGATACGTATACTGTTGG	PCR amplification
#795	CCCAAGCTTGACTATTTAATGATGC	of the *cstH*
#796	CATTTGTACTCGCAATGCTAACTG	Sequencing of the *cstH* from
#797	AATCGTATTTCCAGGATTTTTATCC	Internal sequence of the gene
#814	TTGTGTTGTAATCCTGCTTG	DNA hybridization with *est* cassette
#915	TTACATTTAACCTAGGCGCAACAT	Making $eltB_{44\text{-}64}$cassette
#916	CTTCATCCTTCCTAGGGCTTTTTTTG	with *Sty*I in the ends
#917	AGTCAACATATAGACTCCCAA	DNA hybridization with $eltB_{44\text{-}64}$ cassette

15% (v/v) glycerol, incubated on ice for a further 15 min and then snap frozen in 100 µl aliquots before storing at -80°C.

Fresh competent cells were made essentially as described by Brown *et al.* (1979). A 10 ml LB was inoculated with a single colony and grown overnight at the appropriate temperature. The culture was diluted 1:20 into LB and grown to an A_{650} of 0.6. The cells were chilled on ice for 20 min, pelleted at 4°C in a bench centrifuge, resuspended in 0.5 volumes of ice-cold 100 mM $MgCl_2$, centrifuged again and resuspended in 0.1 volume of ice-cold 100 mM $CaCl_2$ and held on ice for 60 min, before using for transformation.

2.8.2 Transformation

100 µl of competent cells prepared by either procedure (frozen cells were thawed on ice) were mixed with ice-cold DNA (1-2 µg) and incubated on ice for 30 min. The mixture was then heated-shocked at 42°C for 60-90 sec., returned to ice for 2 min., 0.5-1.0 ml of LB was added and incubated at the appropriate temperature for 30-60 min. The mixture was then plated on selection plates.

2.8.3 Electroporation procedure

Electroporation of *E. coli* and ETEC strains was performed by a variation in the method described by Bio-Rad protocol.Briefly, an overnight culture was diluted 1:20 into NB and incubated with shaking until an A_{650} of 0.6, chilled on ice for 30 min and centrifuged at 4°C. The cells were resuspended in one-half volume of cold MQ water, centrifuged again and resuspended in a half volume of ice-cold 10% (v/v) glycerol, centrifuged again and resuspended in a one-tenth volume of ice-cold 10 % (v/v) glycerol.

glycerol. This mixture was kept on ice for 60 min before adding DNA, or stored immediately at -70°C.

Plasmid DNA was added to 40µl electrocompetent cells, mixed and transferred to an sterile ice-cold Pulser™ cuvette (0.2 cm electrode gap, Bio-Rad). Electroporation conditions were 2500 V at 25 µF capacitance and 200 Ω with time-constants of 4.6-4.7 msec. 1.0 ml of LB was added to the cell suspension immediately after electroporation and incubated at 37°C for 1 hr before plating on selection plates.

2.9 Plasmid DNA Preparation precedures

Plasmid DNA was isolated by one of the following procedures.

2.9.1 Large scale purification

Large scale purification of plasmid DNA was performed by the three step alkali lysis method (Garger *et al.,* 1983). Cells from a 1 litre culture were harvested (7,000 r.p.m, 10 min at 4°C) and resuspended in 24 ml of solution I (50 mM glucose; 25 mM Tris-HCl, pH 8.0; 10 mM EDTA). Freshly prepared lysosyme (4 ml of 20 ug/ml in solution I) was added to the cell suspension and incubated at room temperature for 10 min. After addition of 5.5 ml of solution 2 (0.2 M NaOH, 1% (w/v) SDS) the cell suspension was incubated on ice for 5 min which resulted in total lysis of cells. 2.8 ml of solution 3 (50 mM potassium acetate, 11.5% glacial acetic acid) was added, incubated on ice for 15 min and centrifuged at 4°C, 10000 r.p.m for 20 min. The supernatant was collected and extracted with equal volume of (tris-saturated) phenol: chloroform: iso-amyl alcohol (25:24:1) mix. Plasmid DNA was precipitated by addition of 0.6 volumes of propan-2-ol to the aqueous phase and standing at room temperature for 15 min. The DNA was collected by centrifugation at 18,000 r.p.m at 4°C, for 15 min, the pellet was

washed with 70% (v/v) ethanol, dried *in vacuo* and resuspended in 4.8 ml of 1 x TE. Plasmid DNA was further purified from contaminating proteins, chromosomal DNA and RNA by centrifugation at 100 K for 4 hrs, on a CsCl-ethidium bromide gradient. The DNA band was removed by side puncture of the tube with a 19 gauge needle attached to a 1 ml syringe. The ethidium bromide was extracted using iso-amyl alcohol. CsCl was then removed by dialysis of the DNA solution against three changes of 1 x TE at 4°C. DNA was then stored at 4°C or at -20°C.

2.9.2 Small scale plasmid DNA preparation.

Small scale plasmid purification was performed by a modified alkali lysis method of Garger *et al.* (1983). A 1.5 ml of an overnight bacterial culture was centrifuged (2 min, Heraeus Biofuge 15, 15000 r.p.m) and resuspended in 100 µl of solution I. 200µl of solution II was added and incubated on ice for 5 min which resulted in total cell lysis. After the addition of 200µl of solution III, the suspension was incubated on ice for 15 min. Protein, chromosomal DNA and high molecular weight RNA were removed by centrifugation at 15000r.p.m for 20 mins. The supernatant was extracted with tris-saturated phenol:chloroform mix. Equal volume of iso-propanol was added to precipitate the plasmid DNA then left on ice for 15 min, followed by centrifugation for 15 min, at 15000 r.p.m. The pellet was washed with 70% (v/v) ethanol, dried under vacuum and resuspended in 20µl of sterile MQ water or 1×TE pH 7.5.

2.9.3 Preparation of DNA for sequencing by polyethlene glycol precipitation

The method for preparation of DNA is essentially the same as the small scale DNA preparation. After the addition of solution 3 and centrifugation the supernatant was transferred to a new tube. 1µl of Ribonuclease A (RNase A, Sigma) made at 20 µg/

ml was added and the mixture incubated at 37°C for 1 hr. Plasmid DNA was extracted by the addition of a phenol:chloroform:iso-amyl alcohol mixture (25:24:1), precipitated by the addition of an equal volume of 100% (v/v) propan-2-ol and immediately centrifuged for 15 min at 15000 r.p.m. The pellet was washed with 70% (v/v) ethanol and dried *in vacuo*. The DNA pellet was then dissolved in 32 µl of MQ water, followed by the addition of 8 µl of 4 M NaCl and 40 µl of 13% polyethylene Glycol 6000 (BDH). The mixture was incubated on ice for 60 min, then centrifuged at 4°C for 15-30 min. The pellet was washed with 70% (v/v) ethanol, dried *in vacuo* and resuspended in 20 µl of MQ water and stored at -20°C until use.

2.10 Analysis and manipulation of DNA

2.10.1 DNA quantitation

DNA concentration in solution was calculated by measurement of absorption of the solution at 260 nm. It is assumed that an A_{260} of 1.0 is equal to 50µg/ml dsDNA or 33µg/ml of ssDNA.

2.10.2 Restriction endonuclease digestion of DNA

Digestion of DNA with restriction endonuclease was carried out on 0.1-10 µg of DNA in a final volume of 20 µl using restriction enzyme buffers recommended by the manufacturer of the enzyme. DNA was digested in the presence of 0.5 µg RNaseA and 2 units of restriction enzyme per µg DNA. Reactions were incubated at the appropriate temperature for 1-2 hours and were terminated by heating at 65°C for 10 min (except for enzymes with higher inactivation temperatures which were heated to 85°C) prior to

addition of one tenth volume of 15% (w/v) Ficoll, 0 1%(w/v) bromophenol blue, and loading onto a gel.

Partial digestion of DNA was performed by serially diluting (2-fold) the enzyme from a concentration of 0.5u per μg DNA in a solution containing 0.1-0.5 μg DNA in 1 x restriction buffer and incubating at 37°C for 60 min. The reaction was then terminated as described above and partially digested products recovered by gel purification.

2.10.3 Analytical and preparative separation of restriction fragments

Electrophoresis of digested DNA was carried out at room temperature on horizontal 0.8% or 1.0% (w/v) agarose gels (Seakem HGT). Gels were run at 100 V in TBE buffer (67 mM Tris base, 22 mM Boric acid, 1.0 mM EDTA, pH 8.8) or in TAE buffer (40 mM Tris acetate and 2 mM EDTA). After electrophoresis the gels were stained in distilled water containing 2μg/ ml of ethidium bromide for 10-15 min. The DNA bands were visualised by trans-illumination with UV light at 250 nm and photographed using either Polaroid 667 positive film, 665 negative film or a Tracktel gel documentation video imager and thermal printer.

2.10.4 Calculation of restriction fragment size

The sizes of restriction fragments were calculated by comparing their relative mobility with that of *Eco*RI digested *Bacillus subtilis* bacteriophage SPP1 DNA (Ratcliff *et al.,* 1979). The sizes of the SPP1 *Eco*RI digested fragments used were (in kb): 8.5; 7.35; 6.1; 4.84; 3.59; 2.81; 1.95; 1.86; 1.51; 1.39; 1.16; 0.98; 0.72; 0.48; 0.36. (Bresatec).

2.10.5 Gel extraction of DNA fragments and PCR products

Samples of DNA intended to be extracted from agarose gels were loaded, in duplicate, consecutively on 1% agarose gels. After electrophoresis, the gel was cut in two allowing one sample to be visualised by trans-illumination. Once the position of the fragment on the gel had been determined, the gel segments were aligned, the fragment on the untreated portion was excised and the gel slice placed in a dialysis tubing along with 500 µl of sterile TE. The gel slice was electrophoresed for 1 h at 100 V. The DNA solution was extracted with Tris-saturated phenol:chloroform once and precipitated with two volumes of ethanol plus a tenth volume of 3 M sodium acetate, pH 5.0 for ca. 30 min at -70°C. The precipitated DNA was collected by centrifugation at 15000 r.p.m for 15 min, washed with 70% ethanol, dried *in vacuo* and dissolved in TE buffer.

Purification of DNA from a gel was also performed using a QIAEX DNA extraction kit. Products from PCR amplification were isolated both by gel extraction, and by the method provided in the QIA quick-spin PCR purification kit according to the manufacturer's instructions.

2.10.6 Dephosphorylation of DNA

Dephosphorylation of digested DNA was carried out using alkaline phosphatase. For this, the dephosphorylation buffer recommended by the manufacturer was added to the digestion reaction containing 1-2 µg of DNA. One unit of calf intestinal alkaline phosphatase (CIP) was added to the restriction digests that generated 5' overhanging ends, and the mixture incubated at 37°C for 30 min. For digested DNA with 3' overhanging, or blunt ends, 1.0 unit of CIP was added and the mixture incubated at 37°C for 15 min. Then, a further unit of CIP was added and the mixture incubated at 55°C for a further 45 min. In all cases, EDTA, pH 8.0, to a final concentration of 3 mM was

added, followed by heating at 65°C for 10 min, to terminate the reaction. The volume was made up to 0.1 ml with MQ water and the mixture was extracted with TE-saturated phenol (pH 7.5):chloroform:iso-amyl alcohol (25:24:1). The DNA was precipitated with 0.1 volume of 3 M sodium acetate pH 5.2, two volumes of 100% ethanol and incubated at -20°C overnight or at -70°C for 20 min. In the case of small fragments 20 μg of glycogen was added as a carrier. DNA was recovered by centrifugation (15 min, at 15000 r.p.m), pellet washed with 70% (v\v) ethanol and dried *in vacuo*. The DNA was resuspended in an appropriate volume of MQ water or TE, pH 7.5, and stored at -20°C.

2.10.7 Ligation of DNA fragments

Ligations were carried out using 1-2 unit of T4 DNA ligase, and vector DNA and insert DNA at a ratio of approximately 1:5 in a final volume of 20 μl. Ligation mix was incubated for 2 hrs at 16°C or overnight at 4°C for "sticky end" ligations or 30°C for "blunt end'' ligations.

2.10. Cosmid Cloning

The purified plasmid DNA prepared from strain PB176 was partially digested with *Sau*3AI. Samples were taken at specific time intervals, and ligated to pPM2101 which had previously been completely digested with *Bam*HI and dephosphorylated with CIAP. Ligation was allowed to proceed initially at room temperature for 10 h, and then overnight at 4oC. The ligation mix was packaged into bacteriophage λ (Collins and Hohn, 1978) using the *in vitro* Packagene System (Promega) and then transduced into *E.coli* K-12 strain DH1.

Five hundred *E. coli* transductants obtained from cosmid cloning were initially screened by dot blot DNA hybridisation with oligo 299 end-labelled with Dig-dUTP and

terminal transferase (Boehringer Mannheim). Three positive cosmid clones were further characterized by southern hybridisation and one subclone that was positive in this reaction were used for subcloning of *est* gene.

2.10.8 Sequencing using dye-labelled terminators

Plasmid DNA was purified by the method recommended by Applied Biosystems. DNA was sequenced using oligos indicated in Table 2.4, and dye-labelled terminator premix. In a Gene Amp^{TM} reaction tube (0.5 ml, Perkin Elmer) 9.5 µl of premix was mixed with 1-2 µg template DNA and 3 pmol of primer in a final volume of 20 µl and overlaid with a drop of mineral oil and centrifuged briefly.

The reaction mixture was cycled as follows:

Rapid thermal ramp to 96°C

96°C for 30 sec.

Rapid thermal ramp to 50°C.

50°C for 15 sec.

Rapid thermal ramp to 60°C.

60°C for 4 min.

25 cycles total.

Rapid cool to 4°C and hold.

After the cycles were completed, 80 µl of sterile MQ water was added to the reaction tube and the aqueous portion was transferred to a sterile microfuge tube. The DNA was extracted with 100 µl of phenol:chloroform:water (68:14:18). Extraction was repeated again and DNA was precipitated by the addition of one tenth volume of 3M sodium acetate pH 5.2 and two volumes ice-cold 100% ethanol and kept at -70°C for 20

min. The DNA was collected by centrifugation at 15000 r.p.m at 4°C for 30 min. The pellet was washed with 70% ethanol then dried and resuspended in 4.5 µl loading buffer (83% deionised formamide, 8.3 mM EDTA pH 8.0), heated to 95°C for 2 min and loaded onto a 6% polyacrylamide-8M urea gel. The gel was run on a DNA sequencer (Applied Biosystems 373A), the data collected for a period of 13 hrs, analyzed and displayed on the Macintosh IICX as chromatograms.

2.10.9 Sequencing with dye labelled primers

Sequencing was carried out according to the method recommended by Applied Biosystems. 1 µg double stranded plasmid DNA was dispensed into four tubes containing 160ng (tubes A & C) and 320 ng (tubes G & T) of the nucleotides respectively. Dye labelled premixed primer [dyeprimer (0.4 pmol/ml), 5x cycle sequencing buffer, d/ddNTPmix and diluted Taq polymerase (1:8)] was added to the tubes to give a final volume of 5 µl (tubes A & C) and 10 µl (tubes G & T), respectively. Reactions were overlaid with a drop of light mineral oil and subjected to the following cycles:

Rapid thermal ramp to 95°C.

95°C for 30 sec.

Rapid thermal ramp to 55°C.

55°C for 30 sec.

Rapid thermal ramp to 70°C.

70°C for 60 sec.

15 cycles total.

followed by:

Rapid thermal ramp to 95°C.

95°C for 30 sec.

Rapid thermal ramp to 70°C.

70°C for 60 sec.

15 cycles total.

Rapid cool to 4°C and hold.

Samples were combined and 3 µl of 3 M sodium acetate and 100 µl of 95% ethanol added and incubated on ice for 15 min to precipitate the DNA. The DNA was collected by centrifugation at 15000 r.p.m for 15 min. The pellet was washed with 70% ethanol and dried *in vacuo*. Sequencing was carried out as for dye terminator reactions.

2.10.10 Analysis of DNA sequences

Raw sequencing data were analysed by the computer programs, DNASIS and PROSIS (Hitachi Software). Multiple alignments was done using the CLUSTAL V program (Higgins and Sharp, 1989).

2.11 Amplification, and site-directed mutagenesis of DNA:

2.11.1 Polymerase chain reaction (PCR) amplification:

Amplification of DNA fragments through PCR was carried out according to the procedure described by Delidow (1993) for the generation of PCR products with cohesive ends. PCR reactions were performed in GENE-AMP™ reaction tubes (0.5 ml Perkin Elmer) in a 100 µl volume using a thermal cycler (Cetus Perkin Elmer). The reaction mixture contained 1x Taq buffer (50 mM KCl; 10 mM Tris-HCl pH 8.3; 1.1 mM $MgCl_2$; 0.01% (w/v) gelatin), 200 mM each of deoxynucleoside triphosphate (dNTP), 100 pmol each of restriction site-tagged primer, 200 ng of template DNA and 2

units of Taq DNA polymerase (Perkin Elmer). The reaction was overlaid with light mineral oil (Nujol, Perkin Elmer). Amplification was carried out by 25 cycles as follows:

Rapid thermal ramp to 95°C.

95°C for 1 min.

Rapid thermal ramp to 4°C below the T_m of primers.

4°C below the T_m of primers 1 min.

Rapid thermal ramp to 72°C.

72°C for 1 min/1kb DNA polymerisation.

72°C for 10 min.

Rapid cool to 4°C and hold.

Following the PCR the reaction was extracted with an equal volume of Tris-saturated phenol:chloroform mix and the DNA was precipitated as described earlier. Alternatively, the amplified DNA was purified using a Wizard DNA clean up kit (Promega) according to the manufacturer's protocol.

2.11.2 Site-directed mutagenesis PCR

In order to introduce an unique site in the *cstH* gene for inframe insertion of foreign epitopes, the PCR technique was used. The PCR reaction was performed in a GENE AMP™ reaction tube (0.5 ml, Perkin Elmer) in a volume of 100 µl using Vent DNA polymerase (New England Biolabs) and the manufacturer's recommended buffer [10 mM KCl, 20 mM Tris-HCl pH 8.8, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100]. The reaction consisted of 20 ng of template DNA, 200 µM each of dNTP, 100 pmol of primer, 1× reaction buffer and 1 unit of Vent DNA polymerase. The reaction samples were overlaid with light mineral oil (Nujol, Perkin Elmer) and subjected to 25 cycles as follows:

Rapid thermal ramp to 95°C.

95°C for 1 min.

Rapid thermal ramp to 4°C below the T_m of primer.

4°C below the Tm of primer for 1 min.

Rapid thermal ramp to 72°C.

72°C for 1 min/1 kb DNA polymerisation.

72°C for 10 min.

rapid cool to 4°C and hold.

In this PCR the high fidelity of Vent DNA polymerase was employed to minimize the error in the PCR product. Three primers were used in the same reaction, M13 Fwd and M13 Reverse which allowed the amplification of the DNA fragment from the flanking region and the mutant primer which is complementary to the region to be mutagenized. This primer, along with the M13 Fwd primer, produced a fragment of DNA containing the mutation (mega primer). This mega primer was used as primer for further amplification of the full length mutant DNA in the next cycle. The product was a mixture of intact and mutant DNA fragments which, after cloning, was screened for the mutation by restriction digestion analysis. Following the PCR, DNA was purified as described above (section 2.10.5).

2.11.3 Cloning of PCR products

PCR products were cloned by one of the following procedures.

PCR products carrying restriction sites within the primers at ends were digested, purified from the gel and ligated into vector plasmid DNA as described in section 2.10.7.

Alternatively, purified PCR products were directly cloned into the pGEM-T vector (Promega). pGEM-T vector is pGEM-5zf (+) vector which was digested with

*Eco*RV, with an added 3' terminal thymidine at both ends. These single 3'-T overhangs at the insertion site are assumed to improve the efficiency of ligation of a PCR product into the plasmid. Ligation was performed as described previously (section 2.10.7).

2.12 Generation of stepwise deletions:

A set of nested deletions were generated using Erase-a-base kit (Promega) and the method provided by the manufacturer. The 25 μg of plasmid DNA was linearised with the appropriate restriction enzymes before unidirectional digestion of DNA using ExonucleaseIII. In the absence of a protective site, to prevent digestion by ExonucleaseIII (ExoIII), the linear DNA was end-filled with Klenow in the presence of α-Phosphorothioate nucleotides. Digestion with ExoIII was done at 20°C (90 bp/min), samples taken at 20 sec intervals and placed in a tube containing 7.5 μl S1 nuclease mix 940.5 mM potassium acetate pH 4.6, 338 mM NaCl, 1.35 mM $ZnSO_4$, 6.75% (v/v) glycerol containing 2.25 units of S1 nuclease on ice. All samples were incubated at room temperature for 30 min, 1 μl of the stop buffer was added and heated at 65°C for 10 min. The deletion rate was confirmed by electrophoretic analysis of 1ul of each sample, before repairing the staggered ends with Klenow. Samples were then ligated and transformed into *E. coli* K-12 strain DH5α and plated on selection plates. DNA was prepared from transformants and analysed electrophoretically to determine the sizes of each deletion.

2.13 Labelling for restriction fragments and oligonucleotides

Labelling was done using digoxigenin-11-dUTP (DIG dUTP Boehringer Mannheim) according to the manufacturer's protocol. Purified restriction fragments were labelled in a random priming reaction. The DNA fragment to be labelled (1-3 μg) was resuspended in 15 μl of MQ water, boiled for 10 min and then chilled on ice for 2-3

min. A solution containing 2 µl of 1 mM stock of each of the four dNTPs, 0.65 mM DIG-11-dUTP, 142.5 mM Tris-HCl pH 8, 5.7 mM DTT, 14.25 mM $MgCl_2$, 114 mM KCl, 2 µl of hexanucleotide mix and 2 units of Klenow enzyme were added and the mixture was incubated at 37°C overnight. The reaction was stopped by the addition of 2 µl of 0.2 M EDTA pH 8.0. The DNA was precipitated by the addition of 2.5 µl of 4 M LiCl and three volumes of 100% ethanol followed by incubation at -20°C for 2 hrs. Glycogen (20 µg) was added as a carrier. DNA was recovered by centrifugation (15 min at 15000 r.p.m). The pellet was washed with 70% (v\v) ethanol, dried *in vacuo* and resuspended in 20 µl 10 mM Tris-HCl, 1.0 mM EDTA, pH 8.0 and stored at -20°C.

The 3'-end labelling was used to label oligonucleotide probes. Oligonucleotide to be labelled (ca. 200 ng) was mixed with 2.5 µl of 10× tailing buffer (1.4 M potassium cacodylate, 300 mM Tris pH 7.2, 1 mM DTT), 2.5 µl of DIG-11-dUTP, 1.0 µl of terminal transferase and 1 µl of 400 mM $CoCl_2$ and the volume adjusted to 25 µl with the addition of MQ water. The mixture was incubated at 37°C for 60 min, and then stored at -20°C until needed.

2.14 DNA transfer and hybridization (Southern blot)

Electrophoretically separated DNA was transferred from agarose gels to nitrocellulose membranes (Schleicher and Schuell) as described by Southern (1975) and modified by Maniatis *et al.* (1982). The filters were air-dried and then baked at 80°C *in vacuo* for 1-2 hrs. Prior to hybridization with labelled DNA fragment or oligonucleotide probes, filters were incubated for 2 hrs at 42°C in a pre-hybridization solution. The pre-hybridization buffer for a DNA probe contained 50% (v/v) deionized formamide, 50 mM sodium phosphate pH 6.4, 0.75 M NaCl, 75 mM sodium citrate, 5× Denhardt's reagent

(0.1% (w/v) Ficoll, 0.1% (w/v) polyvinylpyrollidone, 0.1% fraction V BSA) and 100 μg/ml single-stranded herring sperm (freshly denatured) DNA. For oligonucleotide probes, the pre-hybridization solution consisted of 1.0 M NaCl, 0.1 M Tris-HCl pH 7.6, 5× Denhardt's reagent, 0.05% SDS, 0.025 M EDTA and 0.1 mg/ml herring sperm (freshly denatured) DNA. For hybridization, the probes were heat denatured for 10 min (100°C for fragment probes), added to the filter in pre-hybridization solution and incubated for 16 hrs at 42°C. Filters hybridized with fragment were washed two times with shaking at room temperature for 5 min with 2× SSC, 0.1% (w/v) SDS, followed by two washes for 15 min at 65°C with 0.2× SSC and 0.1% (w/v) of SDS. For oligonucleotide probes, the filters were washed three times for 10 min in 5× SSC, 0.1% (w/v) SDS, at 5-10°C below the melting temperature (T_m20 of the oligonucleotide (Wood *et al*, 1985)).

After washing, the filters were incubated for 30-60 min in 5% (w/v) skim milk in buffer 1 (100 mM Tris-HCl pH 7.5, 150 mM NaCl). After a brief wash in the same buffer, the filter was incubated with anti-Dig antibody-horseradish peroxidase conjugate at a dilution of 1/5,000 in buffer 1 for a minimum of 30 min. The filters were washed four times for 5 min in buffer 1, followed by two 5 min washes in PBS. The DNA was detected by incubation of the filter with ECL detection reagent (Amersham or Boehringer Mannheim) for 1 min, and exposure of the fiters to X-ray film at room temperature.

2.15 DNA dot blot

DNA dot blots were carried out using a BioRad dot blot apparatus. The DNA was denatured by diluting in 100 μl of 10 mM Tris-HCl pH 8.0, 1.0 mM EDTA, 0.1 M NaCl, adding 6 μl of 5 M NaOH and incubating at 65°C for 60 min. Denatured DNA was neutralized by the addition of 0.1 ml of 3 M NaCl, 0.3 M sodium citrate pH 7.0 and

50 µl of 1.0 M Tris-HCl pH 6.8 and was applied to a nitrocellulose filter. The filter was dried, baked at 80°C for 2 hrs *in vacuo*. Pre-hybridization, hybridization and detection steps were performed as described for Southern hybridization (section 2.14)

2.16 Whole cell lysate

Whole cell lysates were prepared by the procedure which was a modification of that described by Osborn *et al.* (1972). Bacteria were grown in LB (50 ml) at 37°C to an A_{650} of 0.6 and harvested by centrifugation at 10000 r.p.m for 10 min at 4°C. The cells were resuspended in 1 ml of 50 mM glucose, 25 mM Tris-HCl pH 8.0 and 10 mM EDTA and chilled on ice. Bacteria were converted to spheroplasts by the addition of 0.1 ml of 1.0 mg/ml lysozyme in 0.1M EDTA pH 7.3 and incubating on ice for 30 min. The cell suspension was frozen at -70°C for 30 min, then thawed and lysed by 60× 1sec bursts with a Branson Ultrasonifier. Large cell debris and unlysed cells were removed by centrifugation at 5000 r.p.m for 5 min at 4°C. The supernatant (cell lysate) was stored at -20°C until used.

2.17 Preparation of formalin-killed bacteria

Formalin-killed bacteria were prepared by resuspending the CFA grown bacteria in 10 ml PBS. Formalin was added to a final concentration of 1% (vol/vol) and incubated at 37°C for 1 h with occasional shaking. The sterility of the preparations was assessed by plating 200 µl samples onto LB plates and incubating at 37°C overnight. The killed bacterial suspensions were used for boosting injections.

2.18 Pili preparation

2.18.1 Crude pili preparation

2.18.1.1 Method 1

The bacteria from an overnight culture on CFA agar, containing the appropriate antibiotic where necessary, were harvested into 1.0 ml of PBS and incubated at 56°C for 20 min. The cells were removed by centrifugation at 10000 r.p.m for 10 min. The supernatant containing cell appendages was collected, diluted with an equal volume of 2× SDS-sample buffer and used for electrophoresis, or used directly in immunodot blot analysis.

2.18.1.2 Method 2

Bacterial strains were grown on CFA agar plate containing the appropriate antibiotic where necessary, at 37°C overnight. The cells were harvested into 3 ml of PBS and vortexed for 1 min and cells were pelleted by centrifugation at 10000 r.p.m for 10 min. Trichloroacetic acid was added to the supernatant at a final concentration of 5% and incubated on ice for 1 hr. The pili was precipitated by centrifugation at 15000r.p.m for 15 min. The pellets were solublized in 1× SDS-sample buffer and stored at -20°C until needed. The supernatant can also be used without precipitation.

2.18.2 CsCl pili preparation

Large amounts of CS3 pili were prepared from a CS3-producing wild type Enterotoxigenic *E. coli* strain. One millilitre of an overnight culture was plated onto a large CFA agar plate (30×30cm) and incubated at 37°C overnight. The cells were harvested in 50 ml PBS and heated at 56°C for 20 min. After cooling at room temperature the suspension was passed through a 19 g needle. The bacterial cells were

removed by centrifugation at 10000 r.p.m for 10 min. The supernatant was frozen at -20°C, thawed and ammonium sulphate was added to 40% saturation with stirring for 1 hr at 4°C. The precipitated protein was removed by centrifugation at 15000 r.p.m for 20 min at 4°C and resuspended in 2.5 ml 1×PBS. CsCl was added to a final concentration of 40% and the whole was centrifuged for 18 hrs at 35000 r.p.m. The top discreet band containing CS3 was removed and dialysed against PBS overnight. The dialysed solution was layered on top of 40% CsCl in PBS and centrifuged at 35000 r.p.m for 18 hrs. The separate bands in the CsCl gradient were removed, dialysed 4× against PBS at 4°C and checked for purity by PAGE gel electerophoresis with Coomassie Blue and silver staining. The purified pili were stored at -20°C until needed. The most purified sample was used for injecting into the animal for raising antibody against CS3.

2.19 Production of antisera against CS3

CsCl purified CS3 (100 μg) was emulsified in 1 ml of incomplete adjuvant and injected sub-cutaneously at four sites into a rabbit. The animal was subsequently injected with three similar doses of antigen at 14 day intervals. Ten days after the final immunization, a 20 ml blood sample was taken and the serum was separated by incubation at 37°C followed by incubation overnight at 4°C, and centrifugation at 10000 r.p.m for 10 min at 4°C. The serum was stored at -20°C. A working stock of the antiserum containing 0.02% of sodium azide was kept at 4°C. The antiserum was absorbed four times with live PB176p$^-$ (a wild type Enterotoxigenic *E. coli* deficient in CS3) or with bacteria harbouring the plasmid vectors to be used for cloning CS3 or mutant CS3 (5×10^{10} bacteria/ml per absorption). Alternating absorptions were done by incubating the serum at 37°C for 4 hrs or overnight at 4°C. After each absorption the

bacteria were removed by centrifugation and after the final absorption the serum was passed through a 0.2 μm Millipore filter.

2.20 Monoclonal antibody production

2.20.1 Tissue culture solutions

RPMI 1640 powder containing glutamine was dissolved in 750 ml of MQ water and 2 g of $NaHCO_3$ was added. The pH was adjusted with the addition of 2.5 ml of 1 N HCl. HEPES pH 7.2 at final concentration of 15 mM, penicillin at 67 μg/ml and streptomycin sulphate (Sigma) at 100 μg/ml were added to the medium and made up to 1 litre. The medium was filter sterilised (0.2 μm pore size filter) and stored at 4°C. When the medium was used more than 7 days later, glutamine and antibiotics were added again. The medium was supplemented with 10% (vol/vol) heat-inactivated fetal calf serum to support cell growth and J774 cell culture supernatant as feeder cell. HAT (hypoxanthine, aminopterin, thymidine) and HT (hypoxanthine, thymidine) were used as selective reagents at a final concentration of 1:100 from a stock solution consisting of 1.36 mg hypoxanthine, 0.019 mg aminopterin, and 0.388 mg thymidine/ ml.

2.20.2 Immunization of mice to provide immune spleen cells

Female BALB/C mice were used for immunization and preparation of antibody-producing cells. Purified CS3 pili (immunogen) as shown by silver staining of the SDS polyacrylamide gel (fig.3.2) was used for immunization.

50 μg of purified CS3 pili (about 100 μl) was mixed with equal volume of alumina and incubated at 4°C for 1 hr, mixed with 1 ml of incomplete adjuvant and emulsified. The mice were injected at 4 sites subcutaneously with this emulsion three times at two-weekly intervals. Before each boosting the sera of the mice were checked

for production of antibodies by dot immunoblot. At week six, three days before sacrificing, the mice were boosted intravenously with 50 μg of pilus preparation in saline.

2.20.3 Preparation of mouse serum and spleen cells

Three days after final injection the immunized mice were anaesthetised with CO_2, bled from the retro-orbital plexus and sera were prepared as described in section 2.19 and stored at 4°C or -20°C after the addition of sodium azide to a final concentration of 0.02% until used. The sera were used as positive controls for screening of the Mab producing hybridomas by Western blot analysis and ELISA.

For preparation of spleen cells the sacrificed mice were washed briefly with ethanol, and transferred to a sterile Biohazard hood for the rest of the procedure. The spleens were removed from the mice, and cut into small pieces and each spleen was individually homogenized with a loose fitting glass homogenizer in 10 ml of serum-free medium (RPMI 1640). Clumps of cellular debris were removed from the cell suspension and the cells pelleted by spinning at 200 g for 5 min. The cells were washed once in serum-free medium. The red blood cells were lysed by resuspending the cells in 5 ml of Tris-NH_4Cl [1.0 ml NH_4Cl solution (0.83% w/v) and 10 ml of 170 mM Tris-HCl pH 7.65] and incubating on ice for 10 min. The cells were centrifuged again as above, washed in serum-free medium and resuspended in 10 ml of serum-free medium. Cell counts were made using an inverted microscope, after staining with trypan blue. A total of 2 x 10^8 spleen cells in 10 ml were obtained from two spleens.

2.20.4 Thawing frozen cells

Frozen cells from liquid nitrogen storage were thawed ~~at~~ in a 37° C water bath. Equal volumes of pre-warmed medium were added to each vial and allowed to stand for

15 min. The volume of cell suspension was made to 10 ml by the slow addition of medium. The cells were collected by spinning at 200 g for 5 min, resuspended in 10 ml of medium, centrifuged again and, after decanting the supernatant the cells were used for culture.

2.20.5 Preparation of myeloma cell line for fusion

P3-X63-Ag8 6S3 Cells were cultured in 60 ml flasks until the exponential phase of growth. They were then harvested into 50 ml Falcon tubes by centrifugation at 200 g for 5 min and washed once with approximately 30 ml of serum-free medium. After resuspending, the concentration of cells was adjusted to 4 x 10^7 cells/ ml in serum-free medium.

2.20.6 Fusion of myeloma cells with immune spleen cells

All reagents used for making the fusions were warmed to 37°C prior to use. The spleen and myeloma cells were pooled into Falcon tubes at a ratio of 5:1 (spleen cells:myeloma cells). The spleen cells ($2x10^8$) in 10 ml and the myeloma cells (4 x 10^7) in 10 ml were pooled and centrifuged at 400 × *g* for 5 min. After removing 19 ml of the supernatant, the pellet was resuspended, the cells were centrifuged again and the supernatant was removed.

One ml of warm 50% polyethyleneglycol (PEG, the fusing agent) was added to the myeloma/spleen cell pellet slowly over 1 min, gently stirring the cell pellet with the tip of the pipette to resuspend the cells. The cells were incubated at 37°C for 2 min. This mixture was slowly and gently diluted with 2 ml of serum-free medium over 2 min to dilute the PEG. A further 7 ml of serum-free medium was added over 2-3 min gradually diluting the PEG without lysing the cells. The mixture was then centrifuged at

200g for 5 min, the supernatant removed and the cell pellet was resuspended in 50 ml HAT medium (RPMI 1640 containing 10% foetal bovine serum, 10% J774 cell line culture supernatant as feeder cell and 1% HAT).

The cell suspension was plated into a 96-well tissue culture plate (0.1 ml/well) and incubated in a 37°C incubator humidified with 5% CO_2 in air. These plates were the master plates. The day the fusion was performed is referred to as day 0.

2.20.7 Growth of hybrid cells secreting antibody

Two days after fusion, 0.1 ml of HAT medium was added to each well. On day four, the cells in the tissue culture plates were examined under an inversion phase contrast microscope to check for the appearance of hybrid cells and for contamination in the cultures. Most of the hybrid cells were visible at this time. On day 7, the well-grown cultures were transferred into a 24 well tray, 0.5 ml of HAT medium was added and incubation continued. On day 11, 0.5 ml of the supernatant was removed from each well and replaced with 0.5 ml of fresh HAT medium. On days 14 and 17, the hybrid cell colonies in most of the wells were quite large and the colour of the medium had turned yellow. A 100 µl aliquot was taken from each well and used to test for the presence of antibody against CS3 by the enzyme linked immunosorbent assay (ELISA). The A_{450} was measured and an $A \geq 0.2$ above the background was considered as positive

2.20.8 Expansion of positive clones in 6 well trays

The hybrid cells that were considered as positive by ELISA were expanded into 6 well trays. The contents of the well were resuspended and transferred to the wells containing 3 ml of HT medium. It is necessary to grow the cells in HT medium (without aminopterin) for about one week after being in HAT medium. Between 2 and 5 days

after this expansion, 5 ml of HT medium was added to each cell culture. When the hybrid cells were well grown, 5 ml of supernatant were taken for further screening by ELISA and Western blot analysis.

2.20.9 Expansion of clones

Antibody-producing hybrid cell cultures were expanded in 25 cm^2 flask. When the hybrid cells in the 6 well trays were near confluent, they were resuspended and transferred to a 25 cm^2 flask containing 10 ml of fresh HT medium. The cells were grown until the medium turned orange. The cultures were further expanded by transfer to 25 cm^2 flasks and adding fresh HT medium to the flask. When the cells were at a density of 4-5 $\times$ 10^5 cells per ml they were harvested into 50 ml Falcon tubes by centrifugation at 200 $\times$ g for 5 min and preserved in liquid nitrogen.

2.20.10 Preservation of the hybridomas

The viability of the cells to be frozen was checked by observation under the microscope in presence of 0.4% trypan blue. Cells in 30-50 mls of culture medium were pelleted by centrifugation at 200 $\times$ g and resuspended in freezing solution (HT medium containing 5% dimethyl sulfoxide, DMSO). The cells in freezing mix were frozen in 1.0 ml plastic screw cap vials (Nunc Intermed, Denmark) using a controlled rate freezer where the temperature dropped from ambient temperature (25°C) to 0°C at 5°C/ min, then from 0°C to -25°C at 1°C/ min and from -25°C to -100°C at 5°C/ min. After freezing the vials were stored in liquid nitrogen.

2.20.11 Limiting dilution cloning of hybridoma cells

The antibody producing cell cultures were cloned by the limiting dilution method as described byHarlow and Lane (1988).

The hybrid cells used for cloning were 70-90% viable as assessed by trypan blue exclusion. Approximately 230 live cells in 100 µl were added to 4.5 ml of HT medium and 100 µl aliquots (5 cells/well) were plated into 36 wells of a 96 well microtiter plate. Four ml of HT medium was added to the remaining 1.0 ml of the cell suspension and a further 36 wells were plated with 100 µl aliquots (1 cell/well). 1.4 ml of the remaining cell suspension mixed with 1.4 ml of HT medium and used to plate the remaining 24 wells (0.5 cells/well). The plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. After 4 days, 100 µl of HT medium was added to each well and every three days half of the medium in each well was replaced with fresh HT medium. On day 10 the wells were observed under an inverting microscope to check the presence of colonies of the hybridoma cells. The wells from the last dilution containing hybridoma cell colonies were considered as monoclonal antibody producing cells and transferred to 24 well plates and 0.5 ml of HT medium was added to each well and the incubation continued. After a period of growth, the supernatants from these cultures were tested for antibody against CS3 by Western blot analysis. Positive clones were expanded and preserved in liquid nitrogen as described previously (sections 2.20.9 and 2.20.10).

2.20.12 Ig (Immunoglobulin) isotyping of the antibodies

The isotype of the monoclonal antibodies was determined by the Ouchterlony method using goat anti-mouse immunoglobulin (Sigma). Ouchterlony plates were prepared by pouring 10 ml of 1% (w/v) agarose in saline onto glass microscope slides (5 × 7.5cm). After setting the agar, four sets of holes (one hole in center and six around)

were made in each plate. Goat anti-mouse immunoglobulin (5 µl) which reacted specifically with mouse IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, or IgM, was placed into the central well. The outer wells were filled with 10 µl of the concentrated cell culture supernatant being tested. The culture supernatants were concentrated using centriflo CF-25 membrane cones (Amicon corporation, U.S.A.). Slides were incubated at 37°C for 4 hours and then at 4°C for 48 hours in a humid chamber. The slides were soaked in saline for 1hr then blotted to dryness with Whatman paper. This procedure was repeated several times. The slides were stained with 0.1% Coomasie Blue R250 in 10% methanol, 10% ethanol and 7% acetic acid for 30 min, then destained in 10% methanol, 10% ethanol and 7% acetic acid.

2.21 Preparation of antisera and sIg against hybrid CS3 pili

Immunization of mice was performed according to the regimen described by Stevenson and Manning (1985). *S. typhimurium* strain G30 harbouring plasmids encoding CS3 and hybrid CS3 pili were grown overnight on CFA agar containing Cm. Bacteria were harvested in saline and used for oral immunization. Cells also were treated with 0.1% formalin and stored for use in subsequent immunizations.

Groups of 5 mice were orally immunized with 10^{10} live bacteria in 30µl saline after having first been fed 30 µl of 10% saturated $NaHCO_3$. On day 9 and 16, the mice were injected intraperitoneally with 5 x 10^7 formalin-killed bacteria. Five days after the last immunization the blood was taken, pooled and the sera were prepared as above (section2.19). After euthanasia with CO_2, the intestines were removed from the mice, washed throughly with 10 ml saline and turned inside-out. Inverted intestines were incubated at 37°C in 3 ml saline containing 50 mM EDTA and 0.1 mg/ml soybean trypsin inhibitor for 1 hr. The mucous layer was removed from the gut and the solution

containing mucous was incubated at 37°C for 30 min. then centrifuged at 10000 r.p.m for 15 min. Sodium azide (0.01% final concentration) was added to the supernatant and stored at -20°C until needed.

2.22 SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 15% gels using a modification of the procedure of Lugtenberg *et al.* (1975). The samples were solubilized in 2× SDS-sample buffer and boiled for 5 min before loading. Gels were 1.5 mM thick for the anaylsis of both LPS and protein. LPS gels were run at 12-14 mA constant current for 16 hrs, whereas protein gels were electorphoresed at 100 V constant voltage (through the stacking gel), followed by 150 V constant current (through the separating gel) for a total of 4-5 hrs. Protein gels were stained with gentle agitation overnight at room temperature in (w/v) Coomassie Brilliant Blue R250 which was dissolved in 10% ethanol, 10% methanol and 7.5% acetic acid. Gels were destained with several changes of 10% ethanol, 10% methanol and 7 5% acetic acid.

Size markers (Pharmacia) were phosphorylase B (94 kDa), bovine serum albumin (BSA) (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

2.23 LPS analysis

2.23.1 LPS preparation

Cells from 1 ml overnight cultures (ca 2×20^9 cells/ml) were pelleted, resuspended in 50 µl of lysing buffer (2% (w/v) SDS, 4% (v/v) β-mercapto-ethanol, 10% (v/v) glycerol, 1 M Tris-HCl pH 7.6, 0.1% (w/v) bromophenol blue) and boiled for 5 min. Proteinase K (10 µl of a 2.5 mg/ml in lysing buffer) was added and the mixture was

incubated at 55°C for 3 hrs. Samples (20 μl) were boiled for 3-5 min prior to SDS-PAGE electrophoresis.

2.23.2 LPS-specific silver staining

Silver staining of LPS was performed using a modification of the method described by Tsai and Frasch (1982). The SDS-PAGE gel was fixed for 16 hrs in 40% (v/v) ethanol, 5% (v/v) acetic acid, then oxidised for 5 min. with 0.7% (v/v) periodic acid in 40% (v/v) ethanol, 5% (v/v). After oxidation, the gel was washed 4 times (30 min each) with MQ water and then stained for 10 min in a 150 ml solution containing 28 ml 0.1M sodium hydroxide, 0.2 ml ammonium hydroxide [30% (w/v)] and 2 ml of silver nitrate [20% (w/v)]. The stained gel was developed in a solution of 50 μg/ ml citric acid and 0.05% (w/v) formaldehyde for 5-15 min. The gel was washed with MQ water 3 times (10 min each) and the staining was stopped by placing in 500 ml of MQ water containing 20 ml of glacial acetic acid for 10-20 min.

2.24 ELISA

ELISA microtiter plates (96 wells Costar) were coated with purified CS3 (2 μg/ml , 100 μl/well) by incubating the plates at 4° C overnight. Plates were washed four times with PBS containing 0.05% Tween 20 (PBS-Tween) and non-specific binding sites were blocked with 1% BSA in PBS-Tween at 37°C for 1 hr. Culture supernatants (100 μl/well) were incubated in the plates at 37°C for 4 hr. After washing with PBS-Tween, the trays were incubated with goat-anti-mouse immunoglobulin alkaline phosphatase conjugate (1:2500 dilution) at 4°C overnight followed by addition of substrate [p,nitrophenyl disodum (Sigma) (5 mg/ml in 10% diethanolamine, 1 mM $MgCl_2$ pH 9.8)]

and incubated at 37°C for 2-3 hrs. The A_{405} was measured using a Titertek Multiscan ELISA tray reader . $A_{405} \geq 0.2$ above the background was defined as positive.

2.25 Protein analysis

2.25.1 T7 RNA polymerase expression system

The plasmid containing the gene encoding the protein of interest under the control of T7 promoter was transformed into an *E. coli* strain carrying the plasmid, pGp1-2, which contains T7 RNA polymerase under the control of the λ P_L promoter/ repressor (Tabor and Richardson, 1985). Transformants were selected on NA containing Km and Ap at 30°C. Recombinants were identified by restriction digestion analysis. Ten millilitres of LB containing Ap and Km was inoculated with a single colony and shaken at 30°C overnight. The culture was subcultured 1:20 and incubated with constant shaking at 30°C util an A_{650} of 0.6 was reached. Cells were then shaken for 20 min at 42°C (to induce the synthesis of T7 RNA polymerase). Rifampicin was added to a final concentration of 200 μg/ml to inactivate the *E. coli* RNA polymerase, and the incubation continued at 42°C for a further 20 min. The culture was incubated for a further 2 hrs with shaking at 37°C. The pellet, (Eppendorf, 1 min) was resuspended in 60 μl of saline and vortexed, followed by the addition of 60 μl 2 × SDS-sample buffer and boiling at 100°C for 5 min. It was then subjected to SDS-PAGE.

2.25.2 Western blot analysis

SDS PAGE was performed on 15% polyacrylamide gels. A suspension of 10^9 cells was mixed with equal volume of 2× SDS-sample buffer, boiled for 3-5 min and loaded onto the gel (Laemmli, 1970). After electrophoresis at 150 V, the separated proteins were transferred to nitrocellulose filter (Schleicher and Schuell) for 2 hr. at 250

mA in a trans-blot cell (Bio-Rad). The transfer buffer used was 25 mM Tris HCl pH 8.3, 192 mM glycine and 20% (v/v) methanol. Non-specific protein binding sites on the filter were blocked with 5% skim milk in TTBS [20 mM Tris-HCl pH 7.4, 0.9%(w/v) NaCl, 0.05% Tween 20] for 1 hr at RT. The filters were washed in TTBS, and incubated overnight in antibody solution at room temperature. The immunoblot was washed with TTBS three times and incubated with goat-anti-mouse or goat-anti-rabbit immunoglubolin horseradish peroxidase conjugate (1:30000 dilution in TTBS) for 2 hr. at RT. The filters were washed four times (5 min) with TTBS and two times (5 min) in TBS [20 mM Tris-HCl pH7.4, 0.9% (w/v) NaCl] and antigen-antibody complexes were detected by enhanced chemiluminescence (ECL). The filter was soaked in ECL detection reagent (Boehringer Mannheim) for 1 min., drained and exposed to X-ray film (Kodak).

2.25.3 Immunodot blot

Cell lysates or pili preparations from 10^9 cell were plotted onto the nitrocelullose filter. After brief drying, non-specific binding sites of the filter were blocked with 5% skim milk in TTBS. The blot was treated with antibody and detected by ECL as described (section 2.25.2). The dots were defined as positive (+) and negative (-) according to positive and negative controls or the density times area of the dots was measured by AIS™ program for the imaging research Inc, Brock University, Canada.

2.26 Indirect immunoflourescent microscopy

Bacteria from overnight growth on CFA agar were gently resuspended in PBS. Cells were fixed on microscope slides, covered by 50 µl of the appropriate dilution of antibody and incubated at room temperature for 1 hr. After washing with PBS, slides

were incubated for 1 hr. at room temperature with fluorescein-conjugated goat-anti-mouse or goat-anti-rabbit immunoglobulins (Sigma). Slides were washed with PBS and distilled water, dried and embedded with Moviol [0.4 g moviol (Calbiochem), 1g glycerol, 1ml H_2O, 2ml 0.2 M tris-HCl pH 8.4] containing 100 mg/ ml para-phenyldiamin (Sigma) to reduce bleaching. Photographs were taken on Kodak T max 400 film with a microscope camera Olympus C-35 AD-4 mounted on an Olympus BH2 microscope.

Chapter 3

Isolation of monoclonal antibodies against CS3 pili

3.1 Introduction

Monoclonal antibodies (Mab) are powerful immunochemical tools because of their specificity of binding, homogeneity and ability to be produced in continuous culture (Harlow and Lane, 1988). Kohler and Milstein (1975) developed a technique for continuous culture of specific antibody-producing cells. In this technique lymphocytes from immune mice were fused with myeloma cells. These hybrid cells or *hybridomas* can grow *in vitro* and secrete specific antibodies continuously. The cultured cells may be frozen, preserved and recovered later. The cells can also be injected into mice to generate tumors so that large amounts of antibody are produced by the animal which can be recovered from ascites fluid (Harlow and Lane, 1988). Each hybridoma produces an antibody directed against a single antigenic determinant (epitope), a monoclonal antibody. Mabs are ideal reagents for recognizing the epitopes of a molecule and have been used for the analysis of antigenic determinants of many bacterial cell components (Takeda *et al.,* 1993; Kazemi and Finkelstein, 1990; 1991; Ludwig *et al.,* 1985; Lopez-Vidal *et al.,* 1988). Since a cell line is derived from a single hybridoma cell, and thus the antibody produced by these cells is homogeneous, impure substances which are able to elicit a humoral response can be used to prepare Mabs against a desired antigen. Hybridomas secreting monoclonal antibodies specific for a wide range of bacterial antigens have been described (Lopez-Vidal *et al.,* 1988; Lopez-Vidal and Svennerholm, 1990; Takeda *et al.,* 1993; Worobec *et al.,* 1983).

The production of monoclonal antibodies in large amounts has had an extensive impact on clinical medicine, science and industry (Kosmas *et al., 1993*; Valentino *et al.,* 1985; Kim and Goldschmid, 1994). Mabs have been used extensively to study the structure and structure/function relationship of enzymes and proteins as well as for the epitope analysis of antigens (Bogdan and Apicella, 1995; Bowden *et al.,* 1995; Bowden *et al.,* 1995; Conner *et al.,* 1994; Bizub-Bender *et al.,* 1994; Smith-Gill *et al.,* 1984; Tzartos and Lindstrom, 1980; Yan *et al.,* 1995).

This chapter describes the production of a set of Mabs directed against native CS3 fimbriae. The Mabs were used for epitope analysis of the CS3 and in studying its antigenic variation in different host strains.

3.2 Results

3.2.1 Immunization of mice to provide immune serum and spleen cells

Female Balb/C mice were immunized with purified CS3 pili (section 2.20.2). The CS3 pili preparation was relatively pure as shown by SDS-PAGE and Coomassie Blue and silver staining (Fig. 3.1 and 3.2). Before each boosting, the sera from mice were checked for the presence of antibody by the dot immunoblot method using purified CS3 pili.

Three days after the final injection, sera and immune spleen cells were prepared from two immunized mice (section 2.20.3). The sera were found to contain antibodies against CS3 by dot immunoblot method and were routinely used as positive controls for screening of monoclonal antibody producing hybridomas by Western blot analysis and ELISA. A total of 2×10^8 spleen cells in 10 ml were obtained from two spleens.

Fig 3.1 SDS-PAGE analysis of purified CS3 pili: staining with coomassie Blue '

The CS3 pili released by heating at 56°C from CFA agar grown ETEC strain E9034 were precipitated with ammonium sulphate at 40% saturation. The resuspended pellet was centrifuged on a CsCl gradient at 35 K r.p.m for 18hr. Five bands were separated and a 5µl aliquot of each was electrophoresed in SDS on a 15% polyacrylamide gel. The gel was stained for 15min with Coomassie Brilliant Blue R250 and destained with 10% ethanol, 10% methanol, and 7.5% acetic acid. The arrows correspond to CstH protein, the major subunit of CS3 pili.

Lanes:

1- Lower band

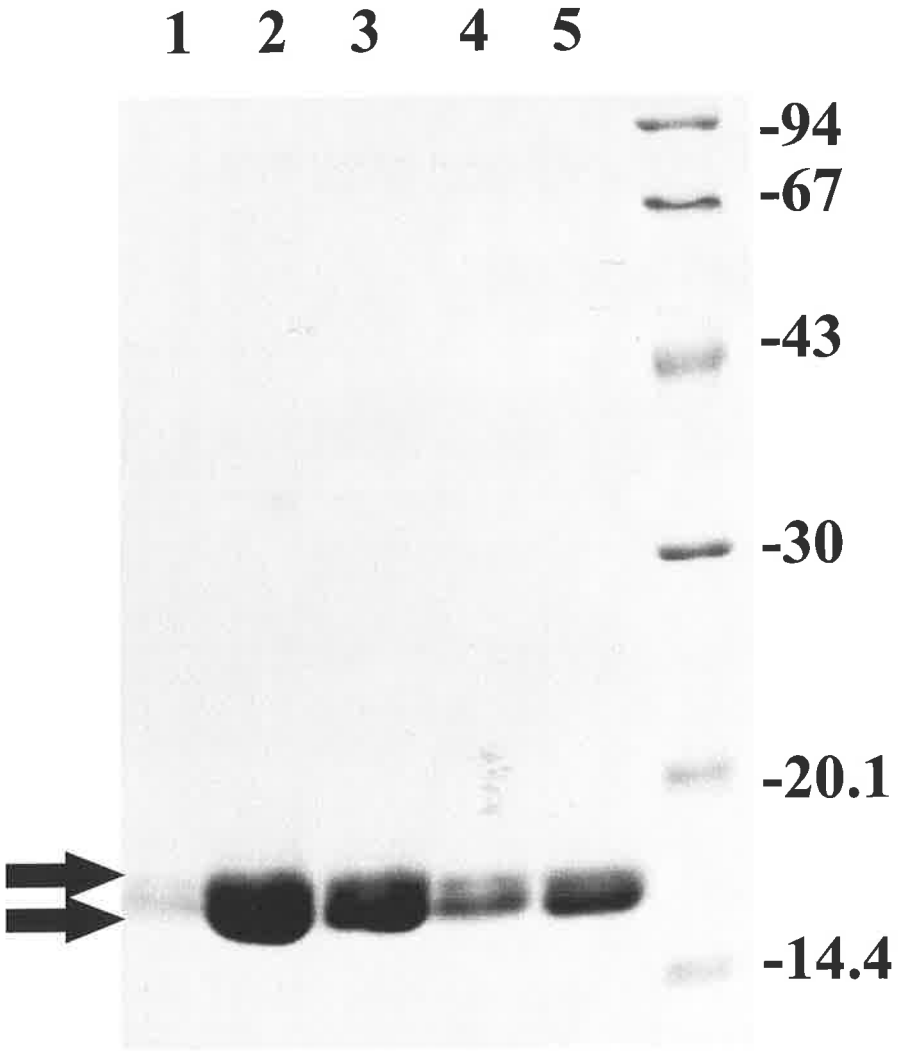
2- Middle band (2)

3- Middle band (1)

4- Top band (2)

5- Top band (1)

MW marker are shown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3
4
5
-94
-67
-43
-30
-20.1
-14.4

Fig 3.2 SDS-PAGE analysis of purified CS3 pili: silver staining

SDS-PAGE analysis of CsCl purified CS3 as per Fig. 3.1.2 μl samples were electrophoresed in SDS on a 15% polyacrylamide gel. After electrophoresis, the gel was silver stained to reveal the LPS content of the CS3 pili preparation. Lanes represent different bands separated in CsCl gradient. The arrows correspond to CstH protein, the major subunit of CS3 pili (lower) and LPS (uppers).

Lanes:

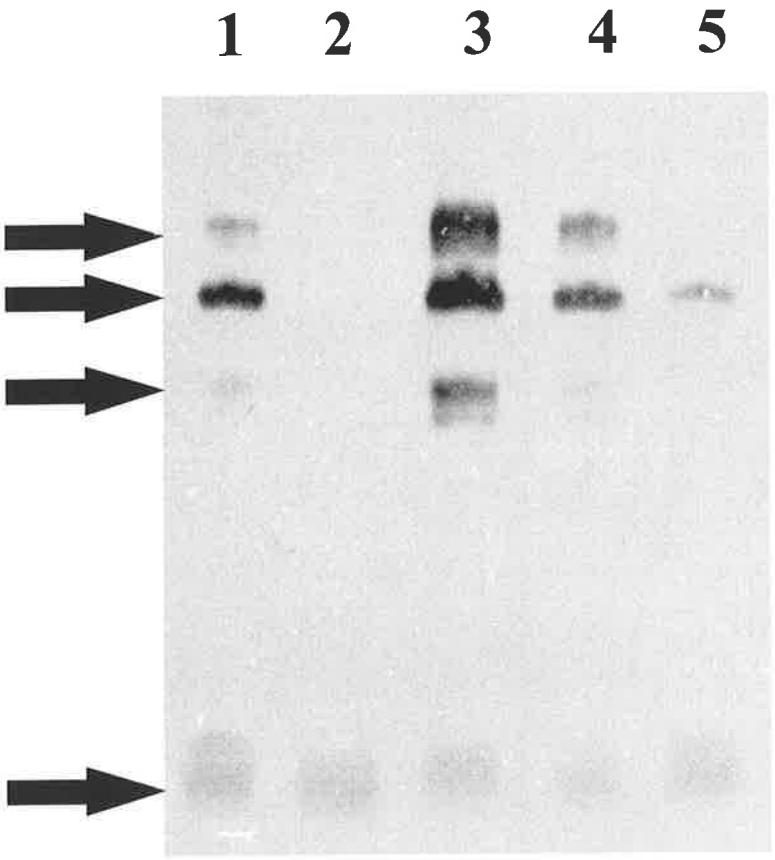
1- Top band (1)

2- Top band (2)

3- Middle band (1)

4- Middle band (2)

5- Lower band


1
2
3
4
5

3.2.2 Fusion of myeloma cells with immune spleen cells

The spleen and myeloma cells were fused as described (section 2.20.6). The hybrid cells were grown in 96 well plates as described in section 2.20.7. A total of 480 well-grown hybrids were transferred into 24 well trays and grown further until the colour of the medium had turned yellow (section 2.20.7). 100µl of medium was taken from each well and used to test for the presence of antibody against CS3 by an enzyme linked immunosorbent assay (ELISA).

3.2.3 Preliminary screening of the hybridoma cells

The hybridomas were screened for antibody production by ELISA using purified CS3 (section 2.24). The A_{450} was measured and an A≥0.2 above the background was considered as positive. From 480 cell cultures, a total of 45 hybridomas had the value of ≥0.2 and these were defined as antibody producing cells and expanded into 6-well trays.

The hybrids that were considered to be positive in the preliminary screening were expanded into 6-well trays (section 2.20.8). When the hybrid cells were sufficiently well grown, 5 ml of the supernatant was taken for further screening by ELISA and Western blot analysis.

3.2.4 Secondary screening of the hybridomas

3.2.4.1 ELISA

From the 45 hybridomas selected by the initial screening, 35 regrew and were defined as positive in the second screening when the culture supernatants were analysed by ELISA. A total of 19 hybridomas with an $A_{450} \geq 0.3$ above the background were selected, called Cst1-Cst19, and used in Western blot analysis to confirm the presence of antibody against CS3.

3.2.4.2 Western blot

Cell lysate from ETEC strain E9034 (producing only CS3) was used to test the Mabs by Western blot analysis. All 19 supernatants reacted with the CstH protein and detected a band corresponding to that detected by the mouse polyclonal antiserum against CS3 (Fig. 3.3). This result indicated that these hybridomas produced antibody against CstH, however, some of the supernatants had multiple reactivites suggesting that they were not clonal.

3.2.5 Limiting dilution cloning of hybridoma cells

Some of the antibody producing cell cultures were cloned by limiting dilution. The hybrid cells used for cloning were 70-90% viable as assessed by trypan blue exclusion. Suspensions containing 230 live cells were used to prepare dilutions for plating into microtiter plates (section 2.20.11). After growing the cells for ten days the wells from the last dilution containing hybridoma cell clones were considered as monoclonal antibody producing cells as shown by Western blot analysis (see following sections) and were expanded.

3.2.6 Characterization of monoclonal antibodies

3.2.6.1 Isotyping of the monoclonal antibodies

The isotypes of the monoclonals as determined by the Ouchterlony method (section 2.20.12) are shown in Table 3-1. The results show that 5 Mabs were IgM, 8 were IgG_1 and four were IgG_{2b}. Two monoclonal antibodies did not react with any of the Goat-anti mouse immunoglobulins. This could perhaps be due to the low concentration of antibodies present in the preparations. The isotypes of the other Mabs were not determined.

Fig. 3.3 Immunoblot analysis of the reactivity of the Mabs with CS3 pili preparation

A whole cell lysate from ETEC strain E9034 was prepared and electrophoresed in SDS on 15% PAGE, transferred to a nitrocellulose filter and cut into strips. The strips were probed with supernatants from hybridoma cultures 1-19. The antigen-antibody complexes were detected by ECL. The arrow indicates the position of the CS3 major pilin subunit, CstH.

Lanes:

1. Mouse polyclonal antiserum to CS3 (+control)
2. Culture supernatant from an ELISA negative culture (- control)

3-21. Culture supernatants from cultures 1-19 corresponding to Mabs Cst1-19

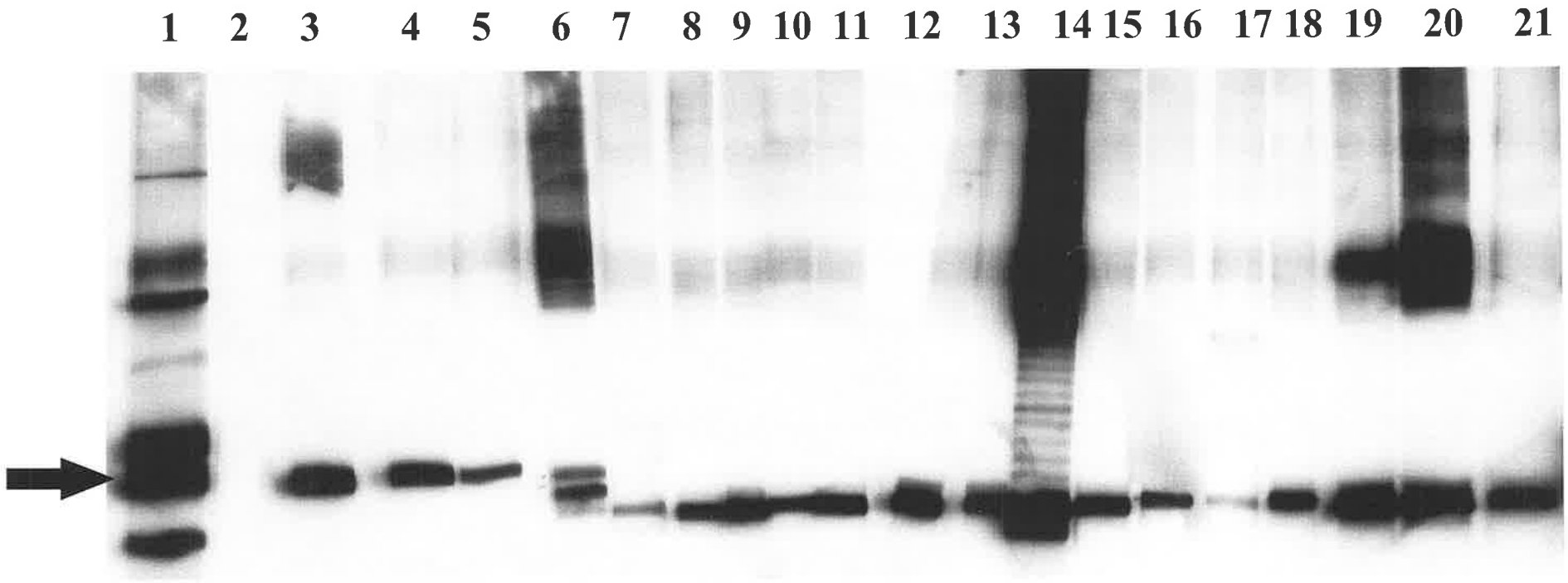

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21

Table 3.1 Characteristics of Mabs

Mab	Isotype	W b/CstH	W b/LPS	I F
CH1	IgG_2	+	-	+
CH2	IgM	+	-	+
CH3	IgG_1	+	-	+
CH4	IgM	+	-	+
CH5	IgG_1	+	-	+
CH6	IgG_1	+	-	+
CH7	IgG_1	+	-	+
CH8	IgG_1	+	-	+
CH9	IgG_{2b}	+	-	+
CH10	IgG_1	+	-	+
CH11	IgG_1	+	-	+
CH12	IgG_{2b}	+	-	+
CH13	IgG_1	+	-	+
CH14	IgG_{2b}	+	-	+
CH15	IgM	+	-	+
CH16	IgM	+	-	+
CH17	ND	+	-	+
CH18	ND	+	-	+
CH19	IgM	+	-	+
CH20	ND	-	+	ND
CH21	ND	-	+	ND
CH22	ND	-	+	ND
CH23	ND	-	+	ND
CH24	ND	-	+	ND
CH25	ND	-	+	ND

W b/CstH: Western blot using cell lysate of E9034. Mabs recognized a band corresponding to CstH
W b/LPS: Western blot using proteinase K treated cell lysate of E9034. Mabs detected bands corresponding to LPS
IF: Immunofluorescence microscopy

3.2.6.2 Specificity of monoclonal antibodies

Immunoblot analysis was performed to define the antigens recognized by the Mabs. 19 Mabs were directed against CstH and six against LPS. Western blot analysis using Mabs CH1 to CH19 revealed a 14-15 kDa polypeptide band corresponding to CstH detected by mouse polyclonal antiserum (Fig 3.4). A whole cell lysate of ETEC strain E9034 was treated with proteinase K and used for characterization of Mabs CH20-CH25. These Mabs could recognize bands in the middle of the gel typical of O-antigen substituted LPS (Fig 3.5).

3.2.6.3 Recognition of CS3 pili by the monoclonal antibodies

Indirect immunofluorescence microscopy and immuno dot blot using pili preparations from wild type ETEC were carried out to check the ability of the Mabs to recognize native CS3 pili on the surface of the bacteria. Mabs CH1-19 could recognize the CS3 pili on the surface of the CS3 positive ETEC strains (Fig 3.6) and in immunodot blot analysis. These Mabs were also able to recognize the CS3 pili on *E. coli* K-12 harbouring the cloned *cst* operon (Fig. 3.7). The results indicate that the Mabs may be useful in diagnostic and epidemiological studies related to CFA/II$^+$ ETEC.

3.3 Discussion

In contrast to the serum from an immunized animal which contains many different antibodies against various antigenic determinants, a monoclonal antibody recognizes a specific epitope of an antigen. Mabs against specific antigenic determinants are also valuable tools as diagnostic reagents, for epitope analysis, for the study of antigenic variation and in epidemiological studies.

Fig. 3.4 Immunoblot analysis of the Mabs directed against CstH

A cell lysate from ETEC strain E9034 was prepared and electrophoresed in SDS on 15% PAGE, transferred to a nitrocellulose filter and cut into strips. Strips were probed with Mabs CH1-19. Antigen-antibody complexes were detected by ECL. The arrow indicates the position of the CS3 major pilin subunit, CstH.

Lanes:

1. Mouse polyclonal antiserum to CS3 (+control)
2. Culture supernatant from an ELISA negative culture (- control)

3-21. Mabs CH1-19

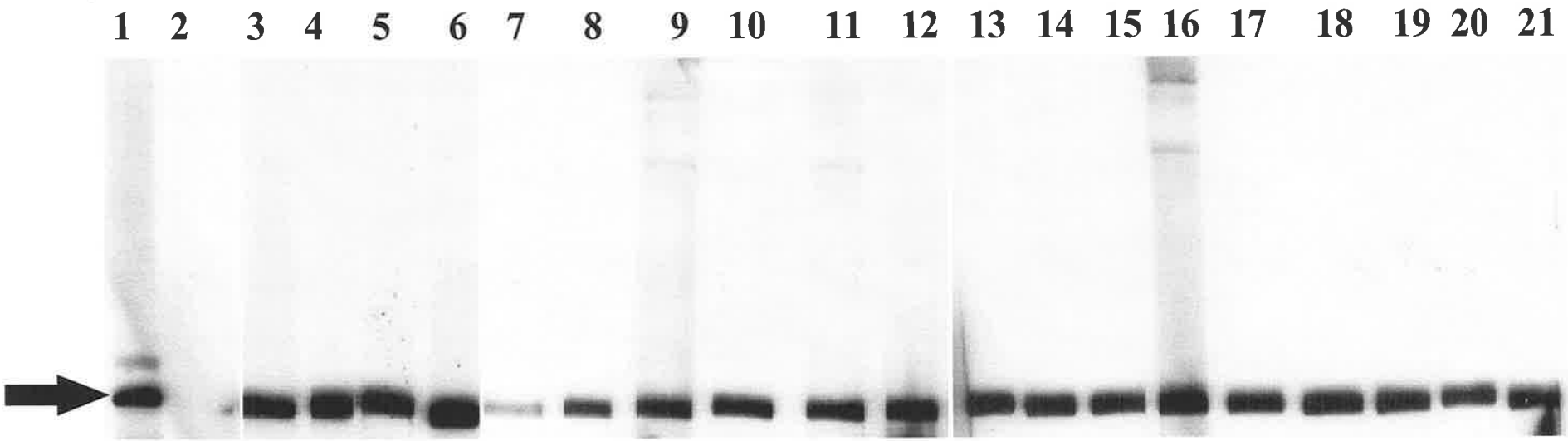

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21

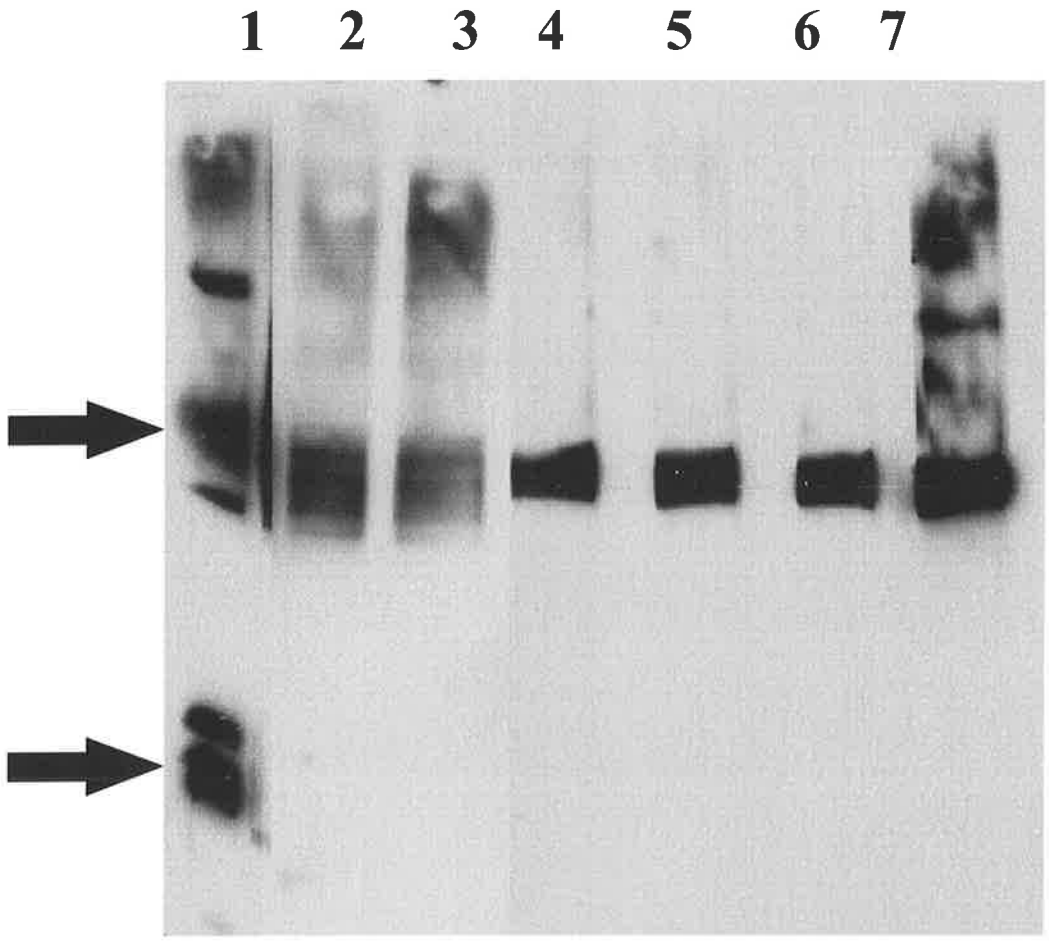
Fig. 3.5 Immunoblot analysis of the Mabs directed against LPS

A proteinase K treated cell lysate from ETEC strain E9034 was electrophoresed in SDS on 15% PAGE, transferred to a nitrocellulose filter and cut into strips. The strips were probed with Mabs CH20-25. Antigen-antibody complexes were detected by ECL. The arrows indicate the position of the CS3 major pilin subunit, CstH (lower) and LPS (upper).

Lanes:

1. Mouse polyclonal antiserum to the CS3 pili preparation. The sample was not treated with proteinase K (+control)

2-7. Mabs CH20-25. Samples were treated with proteinase K


1 2 3 4 5 6 7

A

Fig. 3.6 Indirect immunofluorescence recognition of CS3 pili on the ETEC strain by Mab

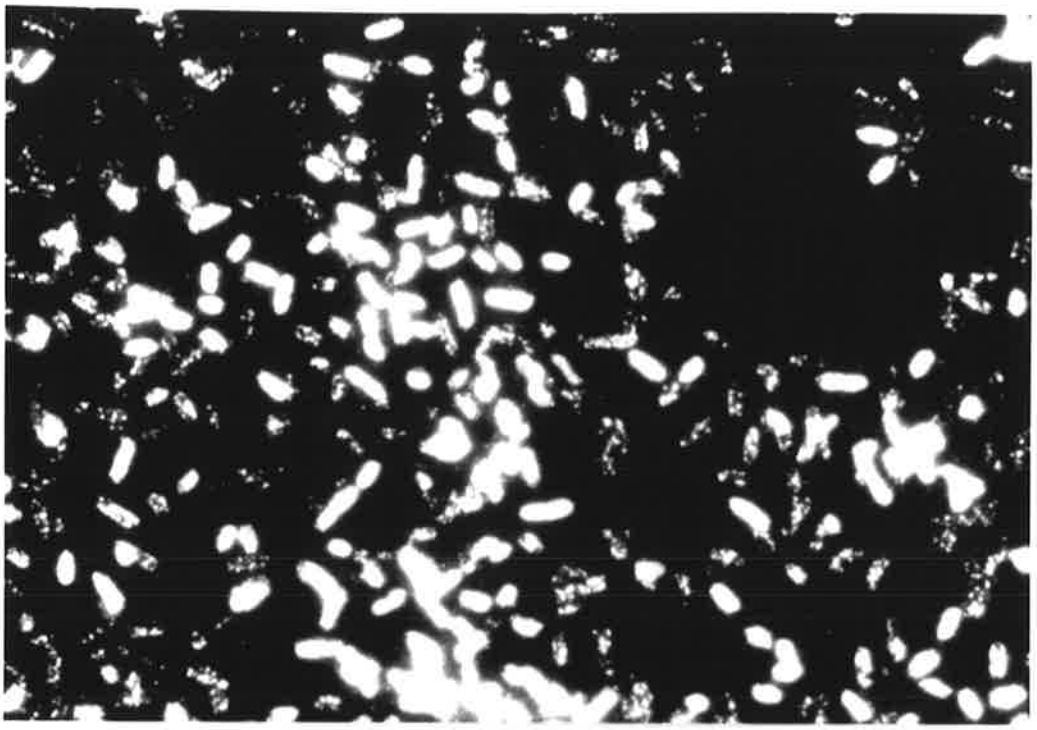
Cells were fixed on a glass slide, incubated with Mab CH1 for 1 hr, washed and incubated with goat anti-mouse immunoglobulin fluorescein conjugate for 30 min. Fluorescent cells were visualized under the fluorescence microscope. The photograph shows the recognition of the CS3 pili by Mab CH1. All other Mabs had the same property.

B

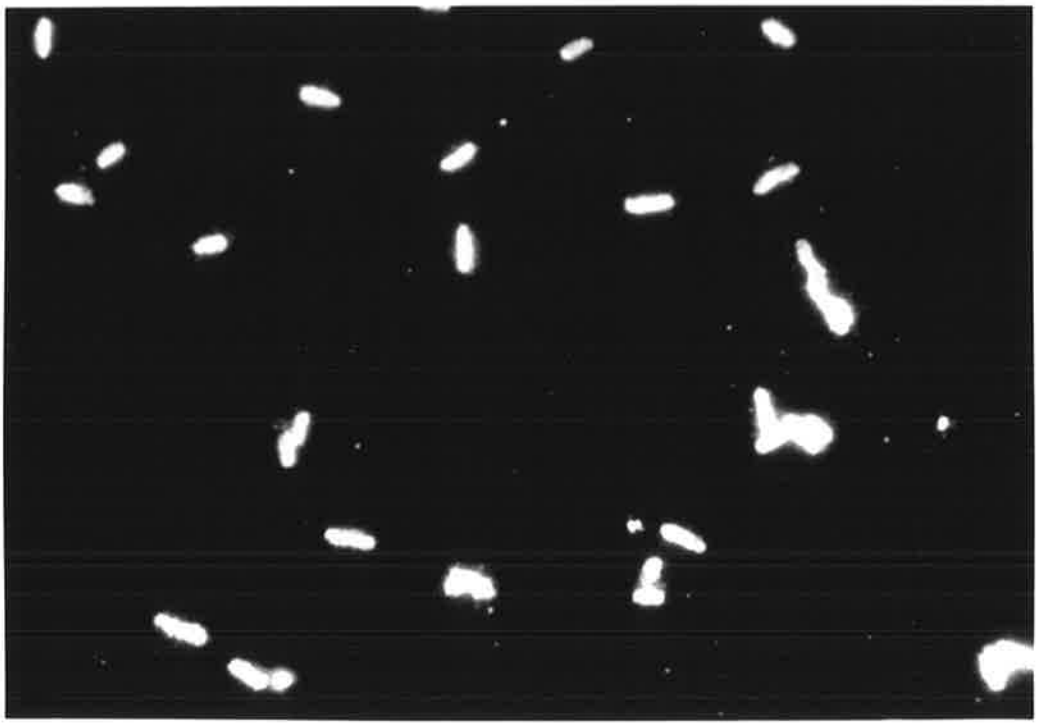
Fig. 3.7 Indirect immunofluorescence recognition of CS3 pili on *E. coli* K-12 strain DH5α (pPM484) by Mab

E. coli K-12 strain DH5α harbouring plasmid pPM484 encoding CS3 pilus biosynthesis was fixed on the slide, incubated with Mab CH1 for 1 hr, washed and incubated with a goat anti-mouse immunoglobulin fluorescein conjugate for 30 min. Fluorescent cells were visualized under the fluorescence microscope. The photograph shows the recognition of the CS3 pili by Mab CH1. All other Mabs had the same property.

A



B



A panel of 19 Mabs against CS3 and 6 Mabs against LPS have been produced in this study. Table 3-1 shows the properties of the Mabs. The Mabs CH1 to CH19 can recognize CS3 on the surface of the bacteria, as indicated by immunofluorescence microscopy and immuno dot blot analysis using purified CS3 pili. They also identify its major subunit, CstH, in Western blot analysis. Immunofluorescence microscopy using a specific Mab is a simple, fast and useful method for the identification of the CS3 subcomponent of CFA/II$^+$ which is known to be expressed by most CFA/II$^+$ ETEC strains. This identification may be important in epidemiological studies to determine the contribution of CFA/II$^+$ ETEC in outbreaks and for assessing the requirements of suitable vaccines in different geographical regions.

In diagnostic and epidemiological studies, it is important to use Mab that can recognize the CS3 antigen in all isolates. In this regard, all anti-CS3 Mabs could recognize CS3 pili on the surface of six ETEC strains expressing different combinations of coli surface (CS) antigens (see Chapter 4) and may be valuable in these studies.

Mabs CH20 to CH25 could recognize bands in the middle of the gel corresponding to the LPS bands. Since the aims of this study did not include the characterization of Mabs against different surface components of the ETEC strains, these Mabs have not been studied in more detail. Further study could be done to characterize the Mabs directed against LPS in detail to identify the exact antigenic determinants for them. These Mabs may be useful in investigations related to the LPS of *E. coli* or other *Enterobacteriacae*.

The main aim of the production of Mabs was to study the variation of CS3 in different ETEC strains from various geographical regions (see Chapter 4). This may be useful in the vaccine development against ETEC. They are also to be used to perform an epitope analysis of CstH for defining the surface-exposed domains on CS3 (Chapter 5).

In this chapter, the production of Mabs against CstH was described and it was shown that these Mabs can recognize CstH protein in immunoblot analysis and CS3 pili on the different CS3 positive strains in immunofluorescence microscopy. Besides the studies reported in this thesis it may also be useful to define the specificity of the Mabs for CS3 and evaluate the potential cross-reactivity with other pili.

Chapter 4

Variation in the CS3 component of CFA/II of human ETEC

4.1 Introduction

Fimbriae are immunogenic and have been used for immunization and protection against ETEC in farm animals (Moon and Bunn, 1993). Immunization with purified CFA/II (Reid *et al.,* 1993; Tacket *et al.,* 1994) or killed CFA/II$^+$ bacteria (Ahren *et al.,* 1993) has been reported to induce the production of antibodies in humans. Therefore it has been proposed that the coli surface antigens (CS factors) or the major antigenic determinants of the CS factors can be used for immunization against CFA/II expressing ETEC. Since CS3 is expressed by most CFA/II$^+$ strains of ETEC (Scotland *et al.,* 1985), CS3 expression and its potential antigenic variation in different host strains is therefore of considerable importance in vaccine development against CFA/II-expressing bacteria.

Fimbriae such as type 1, K88, and P-fimbriae contain conserved regions, which have been suggested to be important for fimbrial biogenesis, and variable domains which represent the antigenic domains, at least partly, of the pili (Van Die *et al.*, 1987; 1988; Dykes *et al.*, 1985; Mooi and de Graaf, 1985; Klemm, 1984). The variable regions of the pili can accept insertion or replacement by foreign epitopes and therefore are good candidates for surface expression of foreign antigenic determinants from other pathogens. Variable domains of P-fimbriae, K88, CS31A and type 1 pili have been used for insertion and expression of various foreign antigenic determinants without any affect

on the biogenesis and assembly of the pili (Bousquet *et al.*, 1994; Hedegaard and Klemm, 1989; Thiry *et al.*, 1989, Van Die *et al.*, 1990; Van der Zee *et al.*, 1995).

Expression of CS3 in different host strains has been studied using two MAbs against CS3 and some antigenic differences in CS3 from different host strains have been suggested (Lopez-Vidal and Svennerholm, 1990). However, there is no information available at the subunit level.

In this chapter the variation in CS3 has been studied in more detail by cloning, sequencing and analysis of the expression of the *cstH* gene from six different strains and by comparing the different CstH proteins with a panel of 21 Mabs. This investigation was performed to evaluate whether CS3 contained any variable domains which might be used for the insertion of foreign antigenic determinants.

4.2 Results

4.2.1 Cloning of the *cstH* genes

Five CFA/II$^+$ strains (Table 4.1) which express different combinations of the CS factors and were isolated from different geographical areas have been used to study the variation in CS3. In order to make comparisons between the various CstH proteins, the *cstH* genes from these strains have been cloned by either PCR amplification or shot-gun cloning.

4.2.1.1 PCR amplification and cloning of the *cstH* genes

DNA fragments containing the *cstH* genes were amplified by PCR from the ETEC strains E41, E9034 and E1392-75. Two oligonucleotides, #794 and #795, based on a known sequence of *cstH* (Jalajakumari *et al.*, 1989) were designed for the amplification of a 650bp DNA fragment encoding the CstH protein. This fragment,

Table 4.1 Properties and origin of the *E. coli* ETEC strains

Strains	Origin	Serotype	Toxin production	CFA
PB-176	Mexico	O6:H16	ST , LT	CS1+CS3
E41	Japan	ND	LT	CFA/II^{+}
E9034	Mexico	O8:H9	ST , LT	CS3
E1392-75	Bangladesh	O6:K15:H16	ST , LT	CS1+CS3
E247425-1	Bangladesh	O6:K15	ST , LT	CS17+CS3
E248750-1	Bangladesh	O6:K-:H16	ST , LT	CS2+CS3

which contains the entire *cstH* gene and its promoter allows the protein to be expressed from its own promoter when cloned in a suitable vector. Oligo #794 corresponding to nt 4082-4104, contains a *Sna*B1 site, and oligo #795 corresponds to nt 4725 to the *Hin*dIII site beyond the end of the *cst* operon with three extra nucleotides added at the 5' end to facilitate cleavage by *Hin*dIII.

The PCR products were purified, digested with *Sna*BI and *Hin*dIII and cloned between the *Hin*dIII and *Sma*I sites of pBluescriptSK (Fig. 4.1). After ligation, the plasmids were transformed into the *E. coli* K-12 strain DH5α. Transformants were screened for the *cstH* gene using the blue/white colour selection on X-gal plates and confirmed by DNA hybridization using the slot blot method with a Dig-labelled 0.65kb *cstH* probe. Several positive clones were further characterized by restriction enzyme analysis. Positive clones were selected from each cloning, having a 0.65kb fragment insert and designated pPM4594, pPM4595, pPM4596, respectively, and used for more detailed study.

4.2.1.2 Shot-gun cloning of *cstH*

The *cstH* genes from two strains, E247425-1 and E248750-1, were isolated by shot-gun cloning. Purified plasmid DNA from these strains was digested with *Eco*RI and *Hin*dIII and cloned between the *Eco*RI and *Hin*dIII sites of pGEM7zf. The ligation reactions were transformed into *E. coli* K-12 strain DH5α and the transformants screened for the *cstH* gene as described above. Some white colonies which were positive in DNA hybridization were further characterized by restriction enzyme analysis. One clone from each cloning, having an approximately 1.7kb *Eco*RI-*Hin*dIII fragment insert, was used for further investigation and designated as pPM4597 and pPM5498 corresponding to E247425-1 and E248750-1, respectively (Fig. 4.2).

Fig. 4.1 Construction of pPM4594, pPM4595 and pPM4596.

Plasmids pPM4594, pPM4595 and pPM4596 contain the *cstH* gene from ETEC strains E41, E9034 and E1392-75, respectively. The *cstH* gene was PCR amplified from these strains, digested with *Sna*B1, *Hin*dIII and ligated to the *Hin*dIII, *Sma*I sites of pBluescript SK. After transformation into DH5α, white colonies on X-gal plates containing Ap were selected and screened for the appropriate insert.

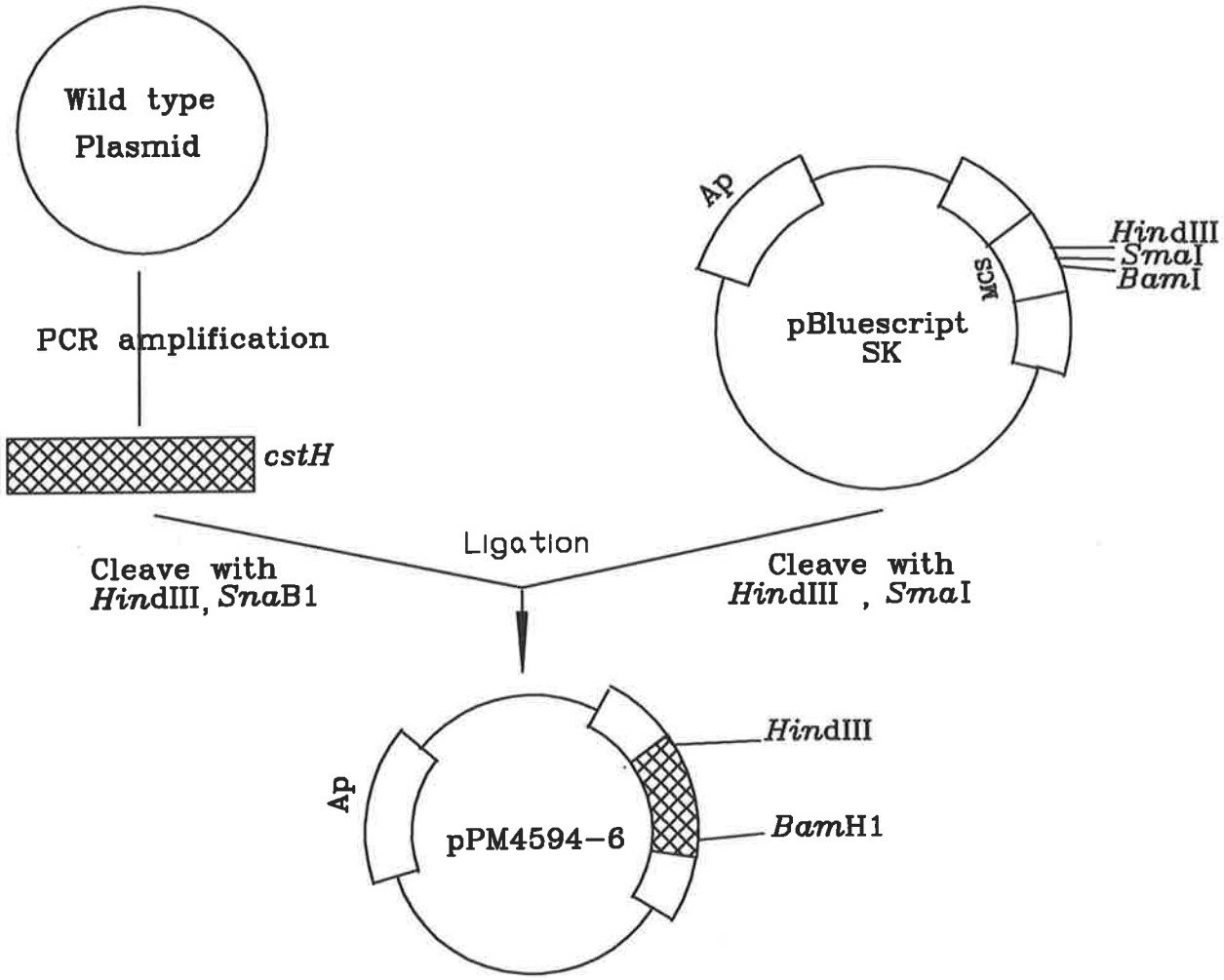
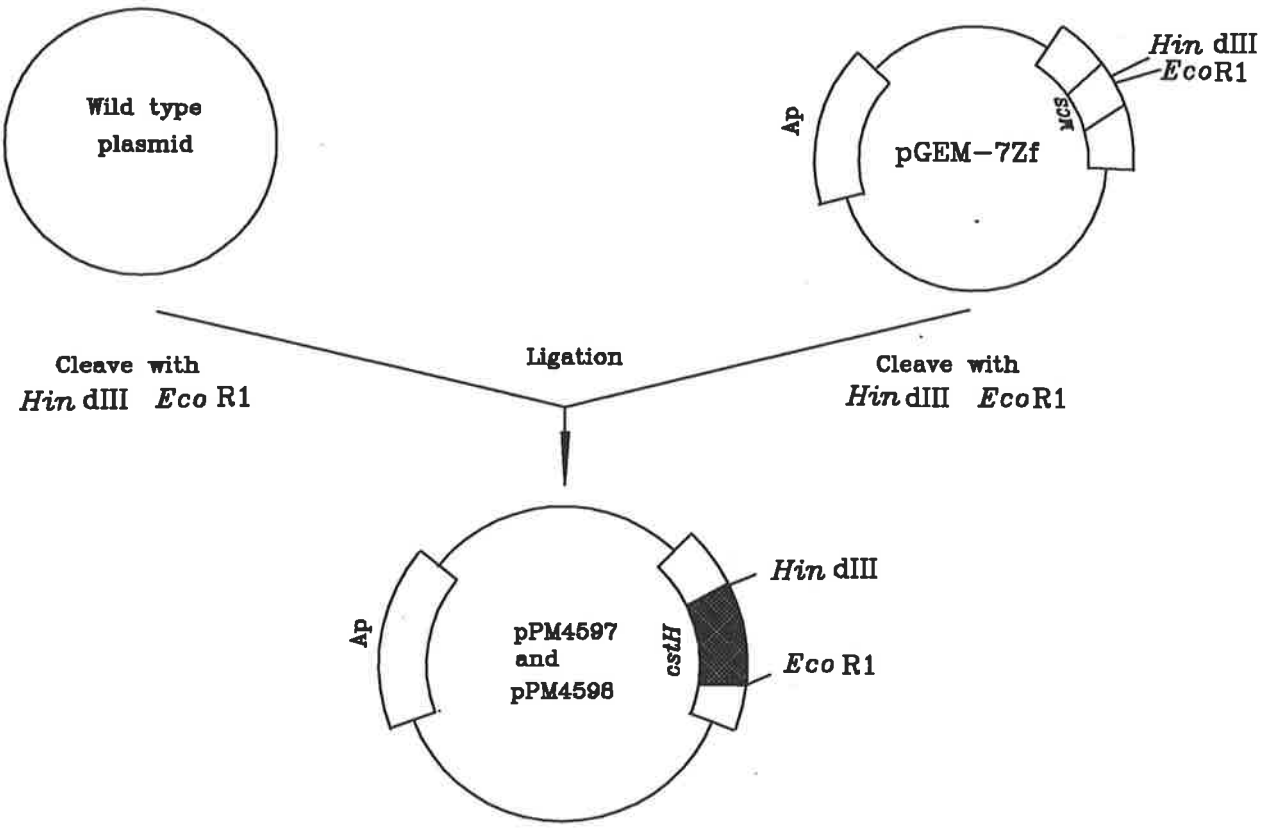

Wild type
Plasmid
PCR amplification
cstH
Cleave with
HindIII, SnaB1
Ligation
Ap
MCS
pBluescript
SK
HindIII
SmaI
BamI
Cleave with
HindIII , SmaI
Ap
pPM4594-6
HindIII
BamH1

Fig. 4.2 Construction of pPM4597 and pPM4598.

Plasmids pPM4597 and pPM4598 contain the *cstH* gene from E247425-1 and E248750-1, respectively. Plasmid DNA from each of the ETEC strains, E247425-1 and E248750-1, was digested with *Hin*dIII, *Eco*RI and the fragments cloned between the *Hin*dIII, *Eco*RI sites of vector pGEM-7zf. After transformation into DH5α, white colonies on X-gal plates containing Ap were selected and screened for the appropriate inserts.


Wild type
plasmid
Ap
pGEM−7Zf
MCS
Hin dIII
Eco R1
Cleave with
Hin dIII Eco R1
Ligation
Cleave with
Hin dIII Eco R1
Ap
pPM4597
and
pPM4598
Hpa
Hin dIII
Eco R1

All of the clones were examined for expression of CS3 by Western immunoblot using a polyclonal antiserum raised against CsCl-purified CS3 pili from E9034. Western blot analysis revealed a band of about 15 kDa (Fig. 4.3) corresponding to the CstH for all subclones.

4.2.2 DNA sequencing and analysis

Sequencing of the cloned *cstH* genes was performed as described in sections 2.10.8 and 2.10.9. The sequencing strategy is shown in Fig. 4.4. The sequences were edited and analysed using SeqEd (Applied Biosystems) and DNAsis (LKB) and alignments performed using CLUSTAL V (Higgins and Sharp, 1989).

The amino acid sequences of the various CstH proteins were predicted from the nucleotide sequences and both the nucleotide and amino acid sequences of all clones were compared with those of *cstH* and CstH from PB176 which were used as a control (Fig. 4.5). A comparison of the gene and amino acid sequences is shown in Table 4.2. The results indicate that there are minor differences between *cstH* from different host strains at both the nucleotide and amino acid levels. However, the degree of conservation is in the order of 99% identity in the sequences.

4.2.3 Variation in the expression of CS3

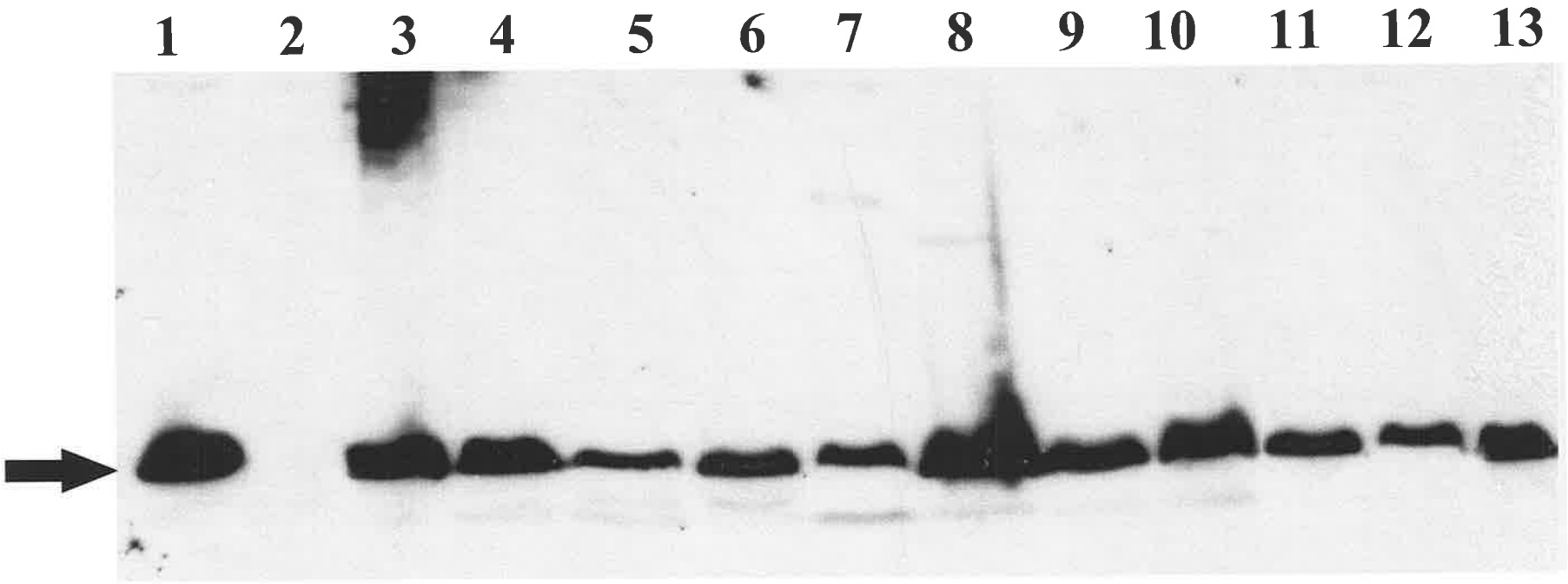
In order to investigate the possible variation in expression of CS3 among the ETEC strains, as suggested by Lopez-Vidal and Svennerholm (1990), the expression of CS3 and CstH were studied by Western blot, immuno dot blot analysis and indirect immunoflourescence microscopy using a panel of 21 monoclonal antibodies and the six CS3/CstH positive enterotoxigenic *E. coli* strains grown on CFA plates. Two of the

Fig 4.3 Western blot analysis of CstH from different strains

Cell lysates of CS3 positive ETEC strains and *E. coli* K-12 harbouring plasmids encoding CstH from different strains were electrophoresed in SDS on 15% PAGE and transferred onto a nitrocellulose filter. A polyclonal antiserum against purified CS3 from E9034 was used as the primary antibody in the Western blot. Antigen-antibody complexes were detected by ECL. The arrow indicates the position of the CS3 major pilin subunit, CstH

Lanes:

1. PB176
2. PB176P$^-$(CS3 negative, as a negative control)
3. E41
4. E9034
5. E1392-75
6. E247425-1
7. E248750-1
8. *E. coli* K-12[pPM4555] expressing CstH from PB176
9. *E. coli* K-12[pPM4594] expressing CstH from E41
10. *E. coli* K-12[pPM4595] expressing CstH from E9034
11. *E. coli* K-12[pPM4596] expressing CstH from E1392-75
12. *E. coli* K-12[pPM4597] expressing CstH from E247425-1
13. *E. coli* K-12[pPM4598] expressing CstH from E248750-1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13

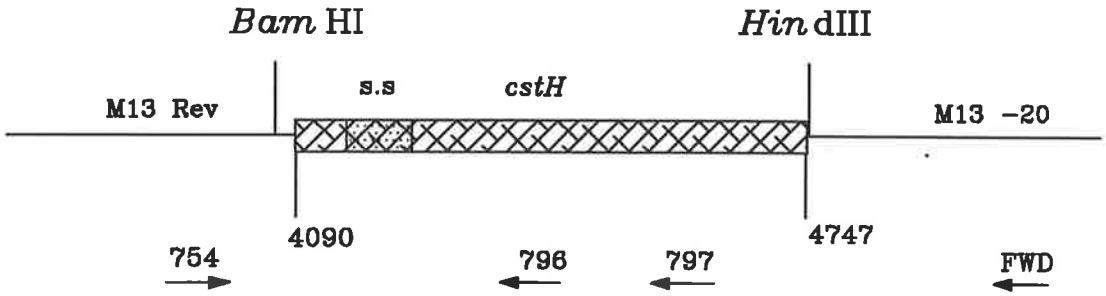
Fig. 4.4 Strategy used for sequencing of the *cstH* genes

The *cstH* genes were cloned as either 0.65 or 1.7 kb fragments. The fragments were sequenced using M13-Reverse primer (oligo #754), M13-Forward primer and the oligos corresponding to the different parts of *cstH* based on published sequenced data for CS3 from PB176 (Jalajakumari *et al.*, 1989). The number above the arrows refers to the oligonucleotides in our laboratory collection. The relevant restriction sites are shown. s.s corresponds to the position of the region encoding the signal sequence of CstH.

Panel A shows the strategy used for the *cstH* genes from strains E41, E9034 and E1392-75.

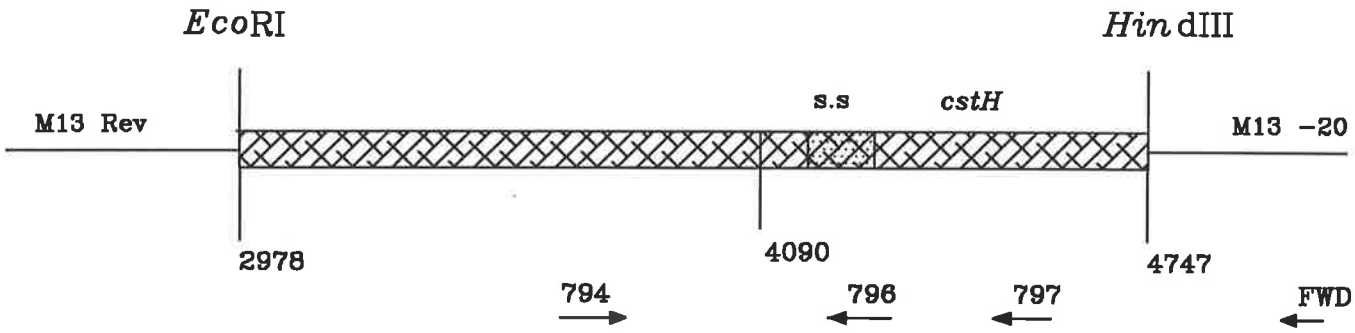
Panel B shows the strategy used for the fragments containing *cstH* genes from strains E247425-1 and E248750-1.

A



pPM4594–6

B



pPM4597–8

Fig. 4 5 Nucleotide and amino acid sequence of CstH

The nucleotide sequence of *cstH* and amino acid sequence of CstH from PB176 has been shown. The signal sequence is indicated as -1 and -22. The mature protein starts from Ala_1

```
  1   CG TAT ACT GTT GGT CTT AAC GTA ACC AGT AAT GTT ATT TAA AGT GAA   47

-22                       Met Leu Lys Ile Lys Tyr Leu Leu Ile Gly Leu  -12
 48  TGT ATG AGG GAT TCG ATG TTA AAA ATA AAA TAC TTA TTA ATA GGT CTT   95
                                             -1   1
-11  Ser Leu Ser Ala Met Ser Ser Tyr Ser Leu Ala Ala Ala Gly Pro Thr   5
 96  TCA CTG TCA GCT ATG AGT TCA TAC TCA CTA GCT GCA GCG GGG CCC ACT  143

 6   Leu Thr Lys Glu Leu Ala Leu Asn Val Leu Ser Pro Ala Ala Leu Asp   21
144  CTA ACC AAA GAA CTG GCA TTA AAT GTG CTT TCT CCT GCA GCT CTG GAT  191

 22  Ala Thr Trp Ala Pro Gln Asp Asn Leu Thr Leu Ser Asn Thr Gly Val   37
192  GCA ACT TGG GCT CCT CAG GAT AAT TTA ACA TTA TCC AAT ACT GGC GTT  239

 38  Ser Asn Thr Leu Val Gly Val Leu Thr Leu Ser Asn Thr Ser Ile Asp   53
240  TCT AAT ACT TTG GTG GGT GTT TTG ACT CTT TCA AAT ACC AGT ATT GAT  287

 54  Thr Val Ser Ile Ala Ser Thr Asn Val Ser Asp Thr Ser Lys Asn Gly   69
288  ACA GTT AGC ATT GCG AGT ACA AAT GTT TCT GAT ACA TCT AAG AAT GGT  335

 70  Thr Val Thr Phe Ala His Glu Thr Asn Asn Ser Ala Ser Phe Ala Thr   85
336  ACA GTA ACT TTT GCA CAT GAG ACA AAT AAC TCT GCT AGC TTT GCC ACC  383

 86  Thr Ile Ser Thr Asp Asn Ala Asn Ile Thr Leu Asp Lys Asn Ala Gly  101
384  ACC ATT TCA ACA GAT AAT GCC AAC ATT ACG TTG GAT AAA AAT GCT GGA  431

102  Asn Thr Ile Val Lys Thr Thr Asn Gly Ser Gln Leu Pro Thr Asn Leu  117
432  AAT ACG ATT GTT AAA ACT ACA AAT GGG AGT CAG TTG CCA ACT AAT TTA  479

118  Pro Leu Lys Phe Ile Thr Thr Glu Gly Asn Glu His Leu Val Ser Gly  133
480  CCA CTT AAG TTT ATT ACC ACT GAA GGT AAC GAA CAT TTA GTT TCA GGT  527

134  Asn Tyr Arg Ala Asn Ile Thr Ile Thr Ser Thr Ile Lys ***          164
528  AAT TAC CGT GCA AAT ATA ACA ATT ACT TCG ACA ATT AAA TAA TTA TAT  575

576  AAT AGA CGT AGC CTT CGA AAT AAA GGC TAC GTT GCT ATC TTT ATG TTT  623

624  GTG ATT TAT AGG CAT CAT TAA ATA GTC AAG CTT                      656
                                        HindIII
```

Table 4.2 Comparison of *cstH* and CstH from different ETEC strains with respect to *cstH* from PB176.

Strains	Seq. differences	Corresponding aa.difference	Overall % identity	
			Nucleotide seq.	Amino acid seq.
E41	$T_{179}\rightarrow C$	$Asn_{109}\rightarrow Asp$	99.4	98.6
	$T_{230}\rightarrow C$	$Thr_{124}\rightarrow Ala$		
	$A_{453}\rightarrow G$			
	$C_{496}\rightarrow C$			
E9034	$T_{305}\rightarrow G$	$Ser_{59}\rightarrow Arg$	99.8	99.3
E1392-75	$T_{292}\rightarrow G$	$Val_{55}\rightarrow Gly$	99.7	99.3
E247425-1	Deletion G_{12}	non-coding	99.8	100
E248750-1	$T_{245}\rightarrow A$	$Asn_{39}\rightarrow Lys$	99.8	99.3

Mabs, 10:2 and 11:2, were those described previously (Lopez-Vidaland Svennerholm 1990). The other 19 Mabs have been described in Chapter 3.

Western blot analysis of these strains using the Mabs revealed a band of approximately 15kDa corresponding to the CS3 major subunit, CstH (Fig. 4.6), which was detected with each of the six different host strains. Some differences were observed in reactivity of the Mabs with the antigens and these are probably due to variation in the concentration of the Mabs in the culture supernatants or the affinity of the Mabs for the antigen. These results indicate that the epitopes recognized by the Mabs are present in all CstH proteins and that the minor amino acid sequence changes did not affect the antibody reaction. However, it needs to be remembered that the epitopes being recognized in the immunoblots are likely to be linear or sequential.

Indirect immunofluorescence microscopy was also used to study the reactivity of the antibodies with CS3 pili, that is assembled CstH. All six strains were recognized by the Mabs in indirect immunofluorescence microscopy with the exception of E248750-1 which did not bind Mab 11:2 (Fig. 4.7). However, Mab 11:2 did react with CstH from this strain in Western blot analysis (see above). In E248750-1, CS3 is co-expressed with CS2 whereas the other strains express either CS3 alone or in combination with other CS factors.

Immuno dot blot using pili preparations from the ETEC strains was performed to check the immunofluorescence microscopy results. Similar results were obtained, pili from all six strains were recognized by the Mabs with the exception of E248750-1 which did not react with Mab 11:2 (Fig. 4.8).

These results indicate that either the expression of CS3 in E248750-1 is different from that in the other strains, that Mab 11:2 is directed against a native epitope

Fig. 4.6 Immunoblot analysis of the Mabs with ETEC strains

Whole cell lysates from the ETEC strains were electrophoresed in SDS on a 15% polyacrylamide gel, transferred to a nitrocellulose filter and cut into strips. The strips were probed with Mabs CH1-19, 10:2 and 11:2. The antibody-antigen complexes were visualized by ECL. The panels show the reactivity of CstH from different ETEC strains with Mabs..

Panel A: ETEC strain PB176

Panel B: ETEC strain E9034

Panel C: ETEC strain E248750-1

Lanes:

1. Mouse anti-CstH polyclonal antiserum (+ control)

2. Supernatant from a negative hybridoma culture (- control)

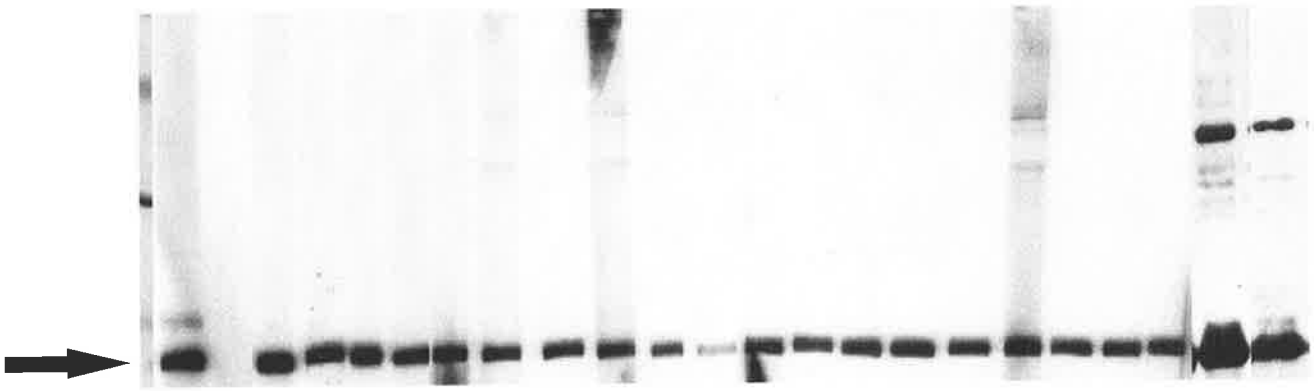
3-23. Mabs CH1-19, 10:2 and 11:2, respectively

The remaining 3 strains (E41, E1392-75 and E247425-1) had the same reactivity with the Mabs

A


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23

B


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23

C

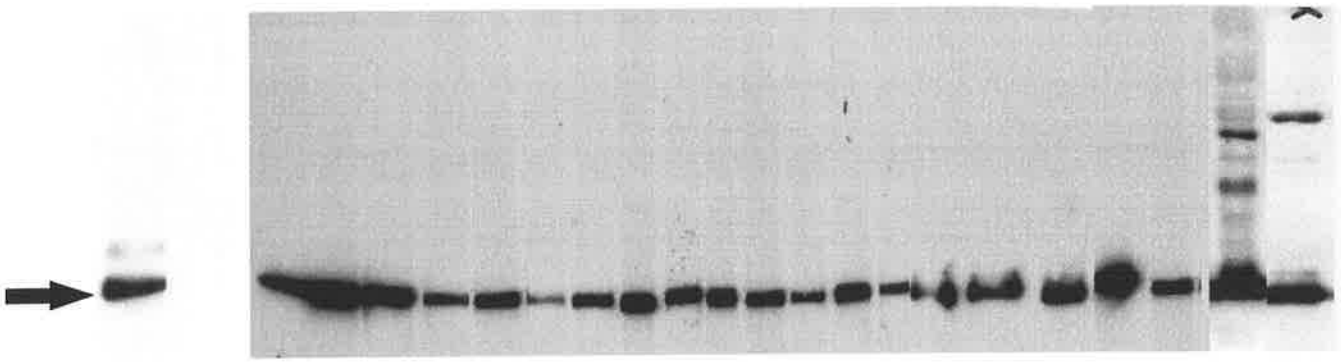

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23

Fig. 4.7 Indirect immunofluorescence recognition of CS3 pili on ETEC strains by Mab

ETEC strain E9034 cells were fixed on the glass slide, incubated with Mab CH1 for 1 hr, washed and incubated with goat anti-mouse immunoglobulin fluorescein conjugate for 30 min. Fluorescent cells were visualized by fluorescence microscopy. The photograph shows the recognition of the CS3 pili by Mab CH1. All other Mabs could recognise all ETEC strains with the exception of Mab 11:2 which failed to recognise E248750-1.

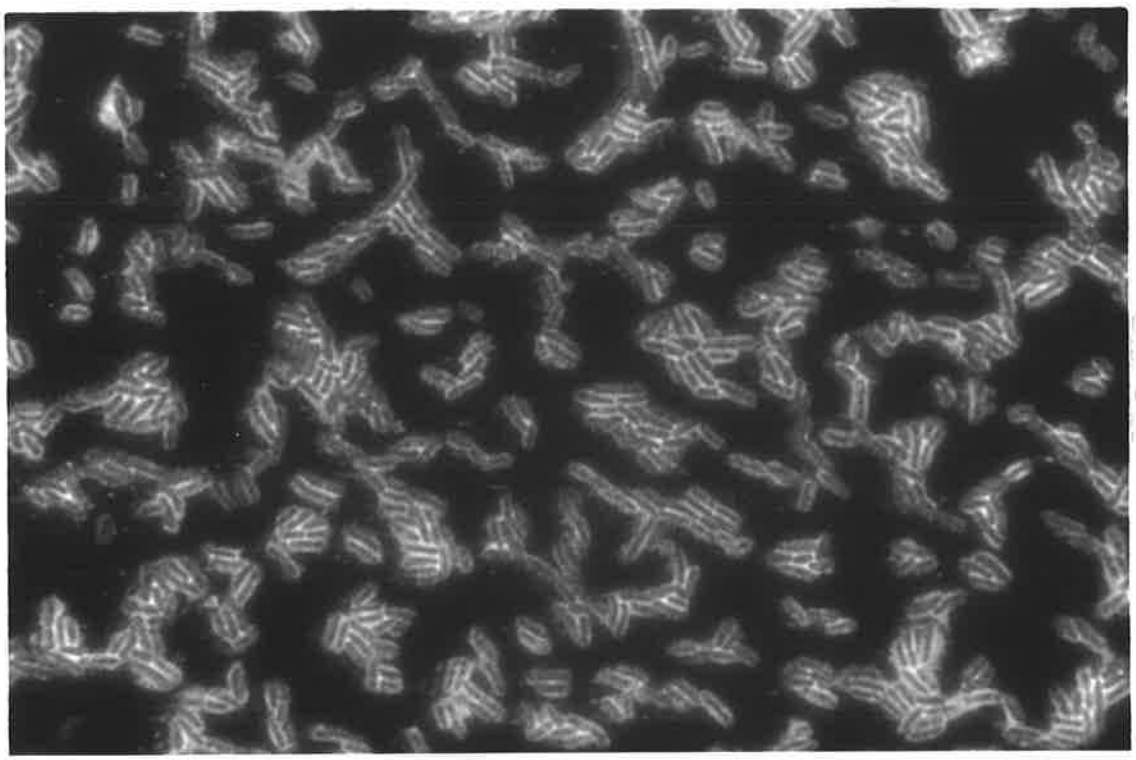


Fig. 4.8 Immunodot blot analysis of CstH from different strains

Pili preparations from six CS3 positive ETEC strains and *E. coli* K-12 harbouring plasmid pPM4740 containing recombinant *cst* operon as well as the control strains were blotted onto nitrocellulose paper, treated with Polyclonal antiserum against purified CS3 from E9034 and 21 Mabs. Antigen-antibody complexes were detected by ECL

Rows:

A. PB176

B. E41

C. E9034

D. $PB176_{P-}$ (CS3 negative, as a negative control)

E. E1392-75

F. E247425-1

G. E248750-1

H. *E. coli* K-12[pPM4740] expressing CstH from E248750-1

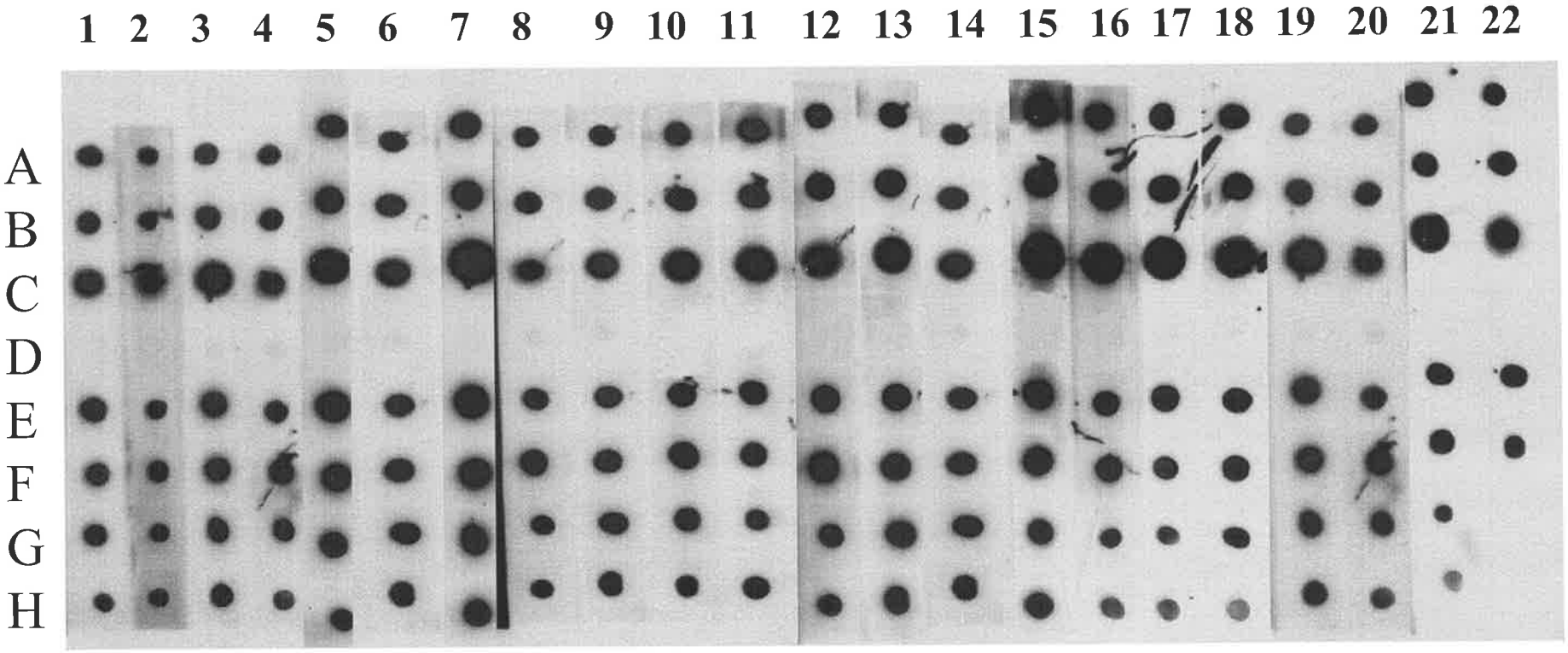
Columns:

1. Polyclonal antiserum against CstH.

2-20. Mab CH1-CH19.

21. Mab 10:2

22. Mab 11:2


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
A
B
C
D
E
F
G
H

of CS3 which is masked by other surface components of the strain or that changes in the amino acid sequence of CstH in this strain prevent recognition by the Mab 11:2.

4.2.4 Cloning of the *cstH* gene from E248750-1 and construction of a heterologous *cst* operon

In order to establish whether the epitope recognized by Mab 11:2 is masked by other surface components of strain E248750-1 or if its expression is influenced by the genetic background of the strain, a recombinant *cst* operon was constructed in which *cstH* from E248750-1 replaced that of PB176. Plasmids pPM4598 (section 4.2.1.2) and pPM484 were digested with *Sna*BI and *Sph*I. A purified DNA fragment from pPM484 containing the assembly gene cluster was cloned in-frame between the *Sna*BI and *Sph*I sites of the plasmid pPM4598 upstream of the *cstH* gene to produce plasmid pPM4740 (Fig. 4.9).

The reactivity of Mab-11:2 and other Mabs with both denatured and native CS3 expressed from plasmid pPM4740 was investigated by immunoblotting and immunofluorescence microscopy. Mab-11:2 showed the same reactivity with CS3 in DH5α[pPM4740] as it did in E248750-1 (Fig 4.8 H and 4.10), suggesting that the failure of this Mab to recognize CS3 pili in this strain is not due to an interaction of CS3 with other surface components of the cell or due to the genetic background of the ETEC strain. Instead, the data imply that it is the differences in amino acid sequences of CstH which are responsible.

The CstH proteins of the six ETEC strains studied differ at five positions (amino acid residues 39, 55, 59, 109 and 124 of the mature protein). The CstH protein of E248750-1 differs from all of the other strains at amino acid #39 having Lys instead of Asn. Thus the reactivity of the various CstH proteins with Mab11:2 in Western blot and

Fig 4.9 Construction of recombinant *cst* operon (pPM4740)

Recombinant plasmid pPM4740 contains the *cstH* gene from E248750-1 and CS3 assembly genes from PB176. pPM484 DNA, harbouring the cloned PB176 *cst* operon was digested with *Sna*BI and *Sph*I. The DNA fragment containing the assembly genes was ligated between the *Sna*BI and *Sph*I sites of pPM4598. Transformants were selected by Ap^R, screened for correct size of DNA and confirmed by restriction enzyme analysis.

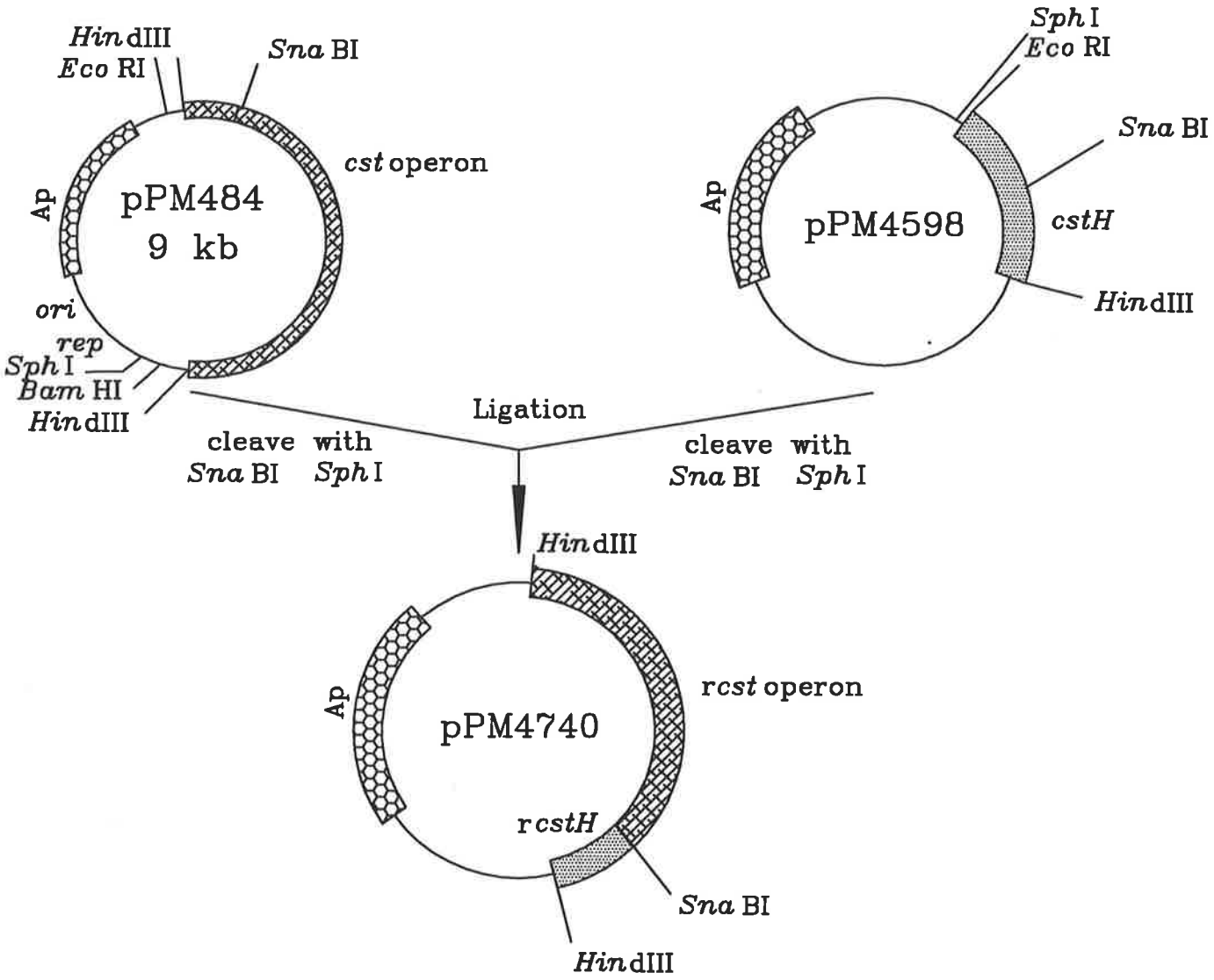
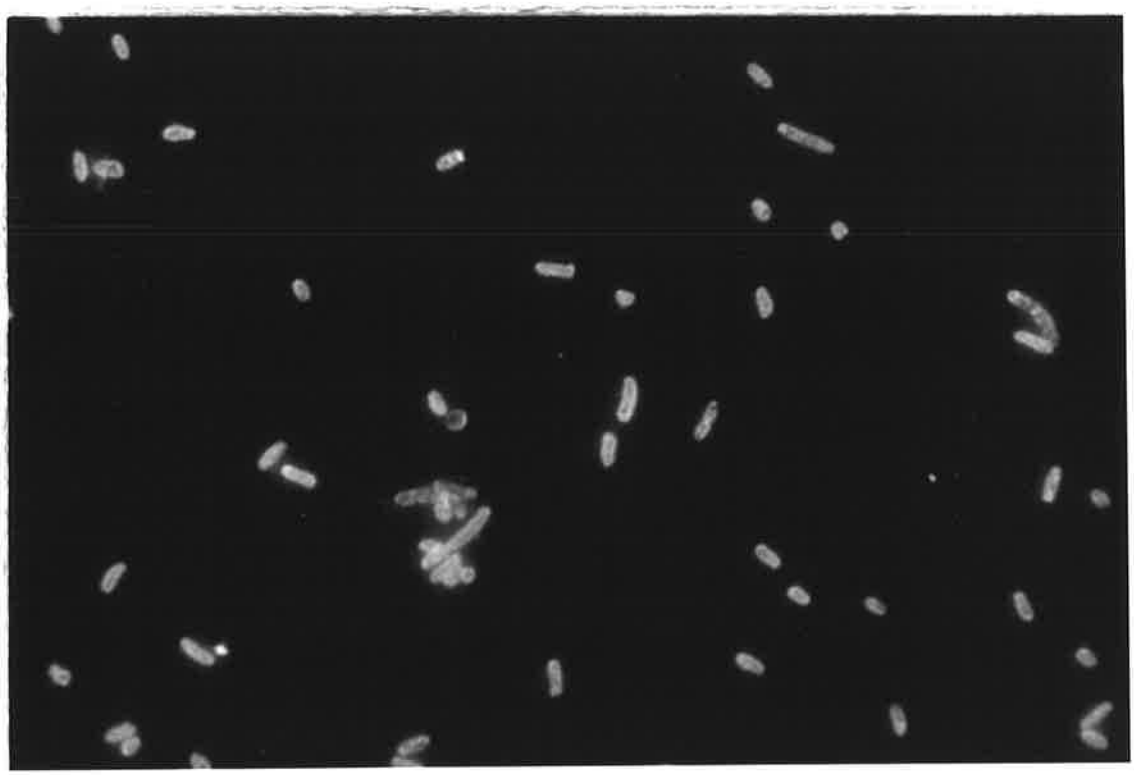

Hin dIII
Eco RI
Sna BI
cst operon
Ap
pPM484
9 kb
ori
rep
Sph I
Bam HI
Hin dIII
Sph I
Eco RI
Sna BI
Ap
pPM4598
cstH
Hin dIII
Ligation
cleave with
Sna BI Sph I
cleave with
Sna BI Sph I
Hin dIII
rcst operon
Ap
pPM4740
rcstH
Sna BI
Hin dIII

Fig. 4.10 Indirect immunofluorescence recognition of CS3 pili on ETEC strains by Mab

E. coli K-12[pPM4740] expressing CstH from E248750-1 was fixed on the glass slide, incubated with Mab CH1 for 1 hr, washed and incubated with goat anti-mouse immunoglobulin fluorescein conjugate for 30 min. Fluorescent cells were visualized by fluorescence microscopy. The photograph shows the recognition of the CS3 pili by Mab CH1. All other Mabs could recognise CS3 pili on this strain with the exception of Mab 11:2 which failed to recognise it.



the lack thereof with CS3 pili on E248750-1 and *E. coli* K-12 expressing CS3 from this strain suggest that this Mab recognizes a conformational epitope whose access to antibody is influenced by amino acid #39. It seems likely that the replacement of Asn_{39} with the positively-charged Lys inhibits the binding of the Mab to the epitope in the assembled pilus, but that denaturation by SDS-PAGE linearizes the protein so that the influence of the Lys is abrogated.

4.3 Discussion

Antigenic variation is the alteration of the antigenic character of the microbial surface often due to mutation in the structural genes encoding the surface components in order to enhance the ability of the microorganism to evade the host immune response (Seifert and So, 1988). Many pili show variation at the amino acid sequence level in domains which are presumed to contain antigenic determinants rather than be involved in the biogenesis of the pili (Van Die *et al.*, 1987, 1988; Dykes *et al.*, 1985b; Klemm, 1984; Mooi and de Graaf, 1985). These variable domains are important in diagnostic and epidemiological studies as well as vaccine development, and have been considered as sites for the insertion and expression of heterologous epitopes (Bousquet *et al.*, 1994; Hedegaard and Klemm, 1989; Thiry *et al.*, 1989, Van Die *et al.*, 1990; Vander Zee *et al.*, 1995).

Variation in the CS3 subunit of six CFA/II^+ strains has been studied by immunoblot and immunofluorescence microscopy and by cloning and sequencing the genes encoding CstH protein. The strains express various combinations of CS factors and were isolated from different geographical regions. 19 Mabs isolated in this study (Chapter 3) and two previously described Mabs (Lopez-Vidal and Svennerholm, 1990) were used. The antigenic variation of CS3 in different ETEC strains presented here

extends and explains the variation in CS3 which has been suggested by Yolanda and Svennerholm (1990). It was observed that although all of the Mabs recognized all of the CstH proteins by immunoblot analysis, Mab-11:2 failed to recognize the CS3 pili from E248750-1 in immunoflourescence microscopy and immunodot blot (Table 4.3).

A similar phenomenon has previously been reported for CFA/I (Lopez-Vidal *et al.*, 1988), CS1, CS2 and CFA/I (Thomas *et al.*, 1985). It has been shown that a Mab reacted with CFA/I in immunoblots but could not recognize the native form of CFA/I on the cell. An antibody against CS4 reacted with CFA/I, CS1 and CS2 in Western blot but could not recognize them in intact fimbriae in either ELISA or immunodiffusion tests (Thomas *et al.*, 1985; McConnell *et al.*, 1989).

The sequencing of the *cstH* genes from six strains showed alterations leading to five amino acid substitutions within the mature protein at residues 39, 55, 59, 109 and 124, and that the CstH protein of E248750-1 is different from the other five strains at amino acid #39 (Lys instead of Asn). When the *cstH* gene from E248750-1 replaced that of PB176 to produce a heterologous *cst* operon that could be expressed in *E. coli* K-12 strain DH5α, Mab 11:2 still failed to recognize the native CS3 produced.

Lopez-Vidal and Svennerholm (1990) showed that Mab11:2 could not recognize CS3 in immunodiffusion but reacted with CstH in immunoblot. These authors suggested that the different reactivity of Mab-11:2 with CS3 was either due to the influence of the biotype of the host strain on the expression of the CS3, or small variations in amino acid sequence of the CstH. The work described here clearly demonstrates that since Mab11:2 failed to recognize the CS3 from E248750-1 produced by *E. coli* K-12, the variation must be independent of the biotype of the strain or the co-expression of the CS3 with the CS2. It was also revealed that CstH from E248750-1 has an amino acid substitution (Asn_{39} →Lys) not found in the other CstH proteins.

Table 4.3 Cross reactivity of Mabs with CstH and CS3 pili from different ETEC strains.

Mab	Immunoglobulin Isotype	W b/CstH	Dot blot/pili	I F
CH1	IgG_2	+	+	+
CH2	IgM	+	+	+
CH3	IgG_1	+	+	+
CH4	IgM	+	+	+
CH5	IgG_1	+	+	+
CH6	IgG_1	+	+	+
CH7	IgG_1	+	+	+
CH8	IgG_1	+	+	+
CH9	IgG_{2b}	+	+	+
CH10	IgG_1	+	+	+
CH11	IgG_1	+	+	+
CH12	IgG_{2b}	+	+	+
CH13	IgG_1	+	+	+
CH14	IgG_{2b}	+	+	+
CH15	IgM	+	+	+
CH16	IgM	+	+	+
CH17	ND	+	+	+
CH18	ND	+	+	+
CH19	IgM	+	+	+
10:2	IgG_1	+	+	+
11:2	IgG_1	+	+ except E248750-1 and DH5α[pPM4740]	+ except E248750-1 and DH5α[pPM4740]

W b/CstH: Western blot using cell lysate from six ETEC strains. Mabs recognized a band corresponding to CstH

Dot blot/pili: Immunodot blot analysis of pili preparation from six ETEC strains

IF: Immunofluorescence microscopy using six ETEC strains

ND: Not determined

Therefore, the only explanation for this phenomenon is the effect of the amino acid change on the antibody recognition. Since Mab-11:2 reacts with the denatured form of CstH in all six strains, the epitope it recognizes must be shared, however, the failure to recognize CS3 in pili on E248750-1 implies that the incorporation of Lys at residue 39 inhibits the binding of the Mab. This suggests that the epitope recognized by this Mab and residue 39 are in close juxtaposition in the CS3 structure. Consequently, determination of the actual sequence of the epitope recognized by Mab11:2 may be useful in CS3 structural studies.

Unfortunately this work did not reveal the presence of any variable domains of CstH which might be potentially useful for the insertion of foreign epitopes. Thus, the most feasible path to follow seems to be to introduce unique sites into regions encoding predicted epitopes of the CstH protein and use them for insertion of heterologous antigenic determinants.

Chapter 5

Epitope analysis of CstH protein

5.1 Introduction

Epitopes or antigenic determinants, are the specific parts of a protein (antigen) that are recognized by antibody or T cells. Identification of epitopes within protein molecules is of importance in the analysis of proteins for function/structure relation studies, understanding the interactions involved in the immune response, and in the design of subunit vaccine and specific diagnostic reagents. Several methods, alone or in combination, have been used for identification of potential epitopes within antigens. These approaches include:

-Prediction of epitopes from the amino acid sequence of a protein (Hopp and Woods, 1981).

-Construction and expression of a sublibrary containing fragments of the known gene with random end points in phage or plasmid expression vectors followed by analyses of the reactivity of the peptides with monoclonal antibodies (Lamb, 1987; Luzzago *et al.*, 1993; Mehra *et al.*, 1986; Scott, 1992).

-Peptide scanning or the investigation of the immunoreactivity of all consecutive overlapping peptides of a known protein with antibodies (polyclonal or monoclonal) against intact antigen (Geysen *et al.*, 1984; 1985; Kohli *et al.*, 1993; Meloen *et al.*, 1986; Proulx *et al.*, 1992; Redlich *et al.*, 1991).

-Deletion mutagenesis from either the 5' or 3' end of the gene and expression of the truncated protein as a fusion protein in a suitable vector and screening with appropriate antibodies (polyclonal or monoclonal) against the intact protein (Gross and Rohrmann, 1990; Skakoan *et al.*, 1993).

-Reactivity of antibodies against the native protein with fragments of the protein produced by CNBr-cleavage (Kahlert *et al.*, 1992), or proteolytic digestion (Mercier *et al.*, 1993; Raman, 1993).

-Raising Mab against synthetic peptides corresponding to predicted epitopes of the protein and using them for epitope mapping of the original protein (Krogfelt *et al.*, 1987; Mercier *et al.,* 1993; Simon *et al.*, 1993; Tahara *et al.*, 1993).

-Site directed mutagenesis (Bakker *et al.*, 1992; Ramanthan *et al.*, 1993; Simon *et al.*, 1993).

-X ray crystallography of the antigen-antibody (Mab) complexes (Prasad *et al.*, 1993).

Fimbriae are essential virulence factors in the pathogenesis of enterotoxigenic *E. coli*. They are immunogenic and vaccines based on fimbriae have been used for protection against animal ETEC. Some fimbriae have also been used for expression of foreign antigens. It seems that the surface exposed domains on the pili represent the epitopes of the protein and are potential sites for insertion and expression of foreign antigenic determinants. In this regard, determination of the exposed epitopes of the carrier protein, in this case the pilin subunit, becomes important in order to define the suitable insertion sites.

In this study, deletion mutagenesis from the N and C terminal coding regions of CstH and fusion of the resulting truncated proteins to a reporter (alkaline phosphatase) has been used for epitope analysis of CstH protein.

E. coli alkaline phosphatase (AP) has been used as a reporter because the sequence of its structural gene, *phoA,* is known. The PhoA protein has a size of 43 kDa which can be easily analysed on standard SDS-PAGE systems. Fusion at its N-terminus should have little effect on the structure of the protein (Hoffman and Wright, 1985). *E. coli* AP is located in the periplasm of *E. coli* K-12 and its export by heterogeneous signal peptides has been reported (Hoffman and Wright 1985). PhoA is stable and can be readily detected on agar medium containing the chromogenic substrate 5 bromo-4-chloro-3 indolyl phosphate (X-pho) (Manoil and Beckwith, 1986). Because of these advantages, *phoA',* lacking the promoter and signal sequence coding region, was chosen to generate CstH::PhoA fusion proteins that are expressed from the *cstH* promoter and contain its signal sequence. *In vivo* expression of the fusions from this promoter resulted in AP activity in bacterial colonies harbouring the in-frame fusion constructs.

5.2 Results

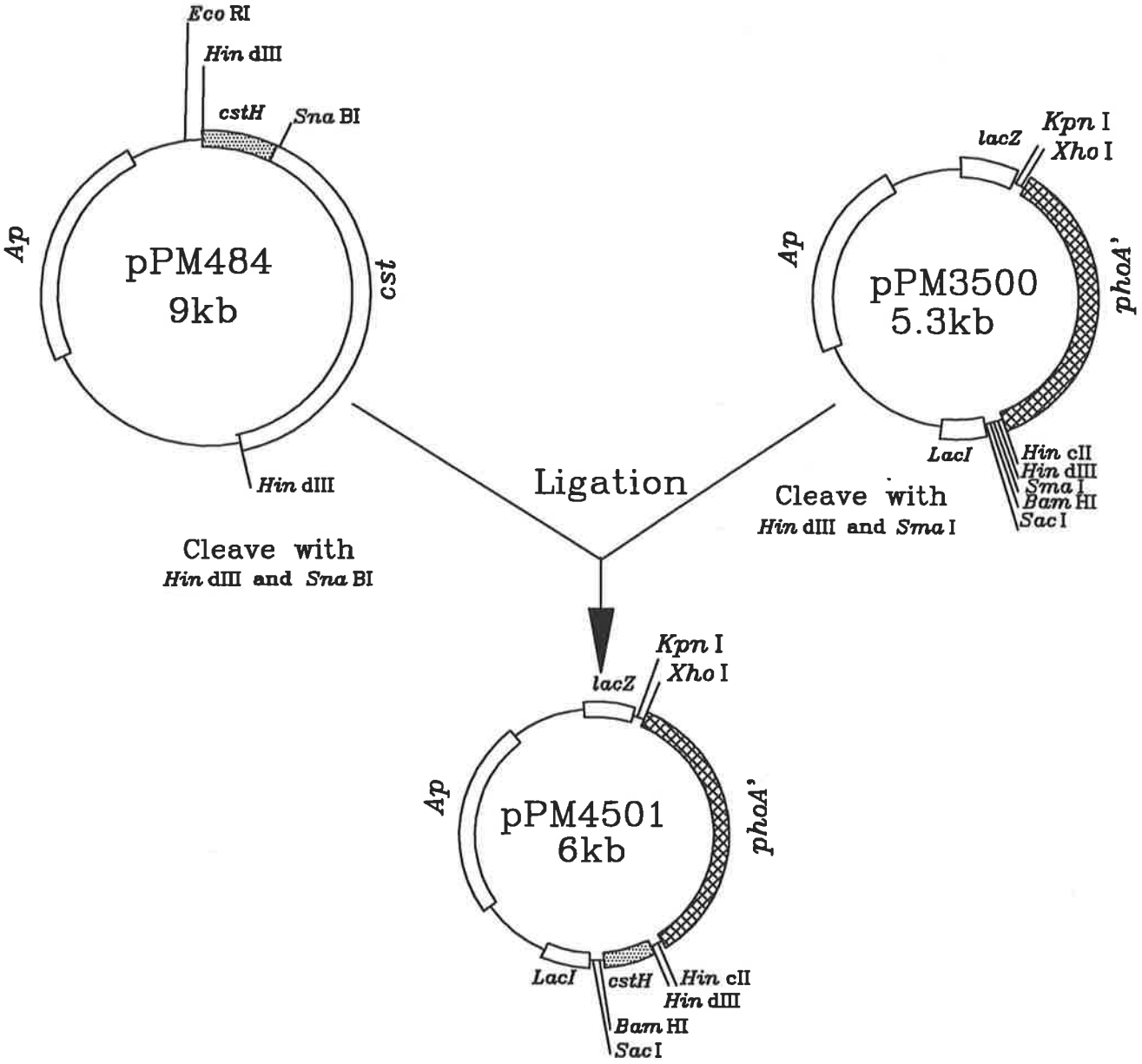
5.2.1 C-terminal deletions in CstH

5.2.1.1 Construction of plasmid pPM4501

The 650 bp DNA fragment encoding CstH from pPM484 was cloned into pPM3500 upstream of the *E. coli phoA* gene lacking promoter and signal sequence (*phoA'*) (Fig. 5.1). The *phoA'* gene is expressed to produce active AP when fused in-frame downstream of a gene having both promoter and signal sequence coding regions. The *cstH* gene has these properties and its in-frame fusion to *phoA'* leads to expression of alkaline phosphatase (AP). Transformants were screened by restriction enzyme analysis and one clone of the right size selected. The junction of the fusion and orientation of the genes was confirmed by sequencing. This clone was designated pPM4501 and used for deletion mutagenesis. The clone contains *cstH* and *phoA'* with

Fig 5-1 Construction of *cstH::phoA'*

The *cstH* gene from the *cst* operon in pPM484 was isolated by digestion with *Hin*dIII and *Sna*BI and the 650bp fragment was cloned between the *Hin*dIII and *Sma*I sites of pPM3500 upstream of *phoA'*. Transformants were selected by Ap^R and screened by restriction enzyme analysis. This plasmid was designated pPM4501.


Eco RI
Hin dIII
cstH
Sna BI
Ap
pPM484
9kb
cst
Hin dIII
Cleave with
Hin dIII and Sna BI
Ligation
Kpn I
lacZ
Xho I
Ap
pPM3500
5.3kb
phoA'
LacI
Hin cII
Hin dIII
Sma I
Bam HI
Sac I
Cleave with
Hin dIII and Sma I
Kpn I
lacZ
Xho I
Ap
pPM4501
6kb
phoA'
LacI
cstH
Hin cII
Hin dIII
Bam HI
Sac I

pPM4501 and used for deletion mutagenesis. The clone contains *cstH* and *phoA`* with suitable cleavage sites in between for unidirectional deletion into *cstH* using Exonuclease III (ExoIII) (Fig 5.1).

5.2.1.2 3` Deletion

A nested set of deletions into the CstH coding region was generated from the 3' end of the *cstH* gene using ExoIII. Two restriction sites, *Hin*dIII and *Bst*BI, were used for deletion. The *Hin*dIII site was protected against exo III by filling with α-phosphorothioate nucleotides and *Bst*BI site used for deletions from 3' end of the *cstH* gene. The DNA from sequential deletion time points was religated and transformed into *E. coli* K-12 strain DH5α. Transformants were screened for the in-frame PhoA fusions on X-pho agar medium. In-frame fusion proteins can be expressed and translocated to the periplasm (where AP is active) by means of the CstH signal sequence, resulting in blue colonies. Blue colonies (AP^+) were further characterized by restriction enzyme analysis (Fig. 5.2, panel A). A total of 13 subclones with different size regions of *cstH* were sequenced in order to precisely define the junction in the fusions. The constructs were designated pPM4502-pPM4514.

5.2.1.3 Expression of fusion proteins

In the first screening of the fusion proteins, 9 subclones were characterized. All of the hybrid proteins could be detected by Western blot analysis using antibodies to AP (Fig. 5.3) but only 8 hybrid proteins could be recognized by the CS3 antibody (Fig. 5.4).

More subclones were screened later to find others with end points at additional sites within *cstH*. Four more clones were characterized giving a total of 13 clones with

Fig 5.2 Analysis of *cstH::phoA* gene fusions by restriction endonuclease digestion.

Plasmid DNA containing *cstH::phoA* hybrid genes was digested with restriction endonucleases *Bam*HI and *Hin*cII (panel A) and *Dra*I (panel B) and electrophoresed on a 1.4% agarose gel. The small variable fragments correspond to deletions of *cstH* (arrow). SPP1 phage DNA digested with *Eco*R1 was used as a size marker. The arrows indicate the smallest fragment.

Panel A: C-terminal deletions. DNA digested with *Bam*HI and *Hin*cII.

Lanes:

1. Size markers.

2-13. *cstH::phoA* hybrid genes.

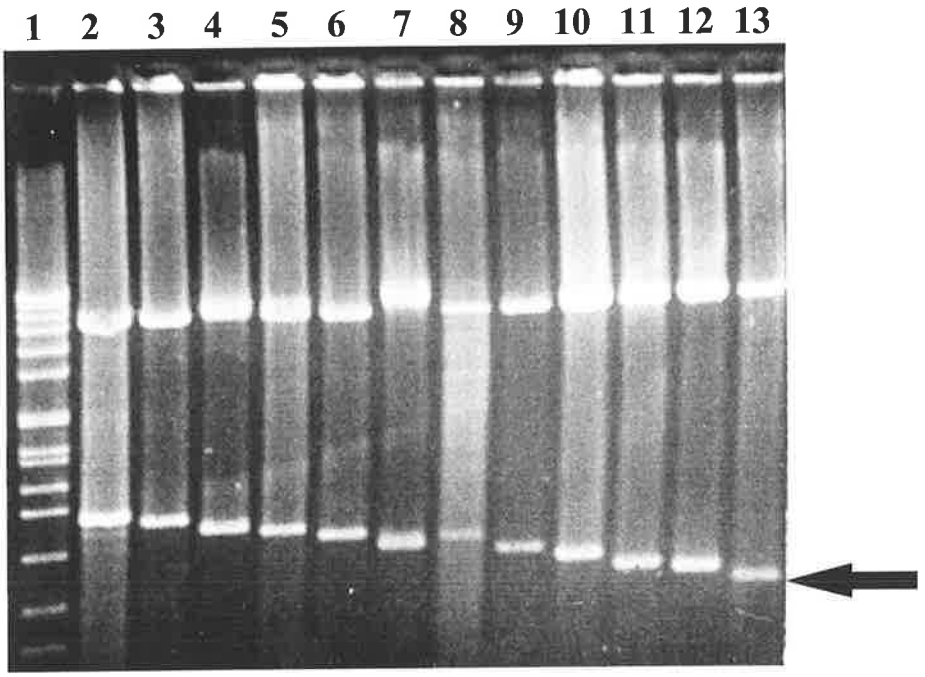
Panel B: N-terminal deletions. DNA digested with *Dra*I.

Lanes

1. Size markers

2-18 *cstH::phoA* hybrid genes.

A



B

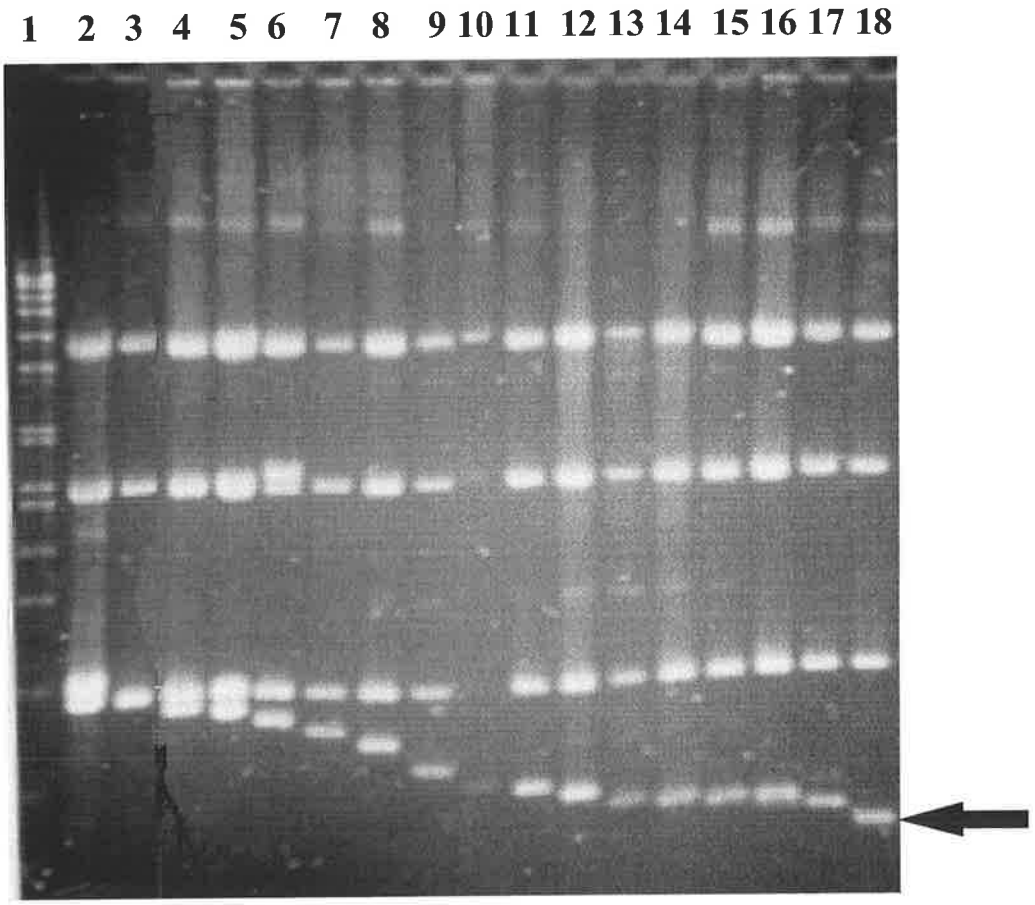
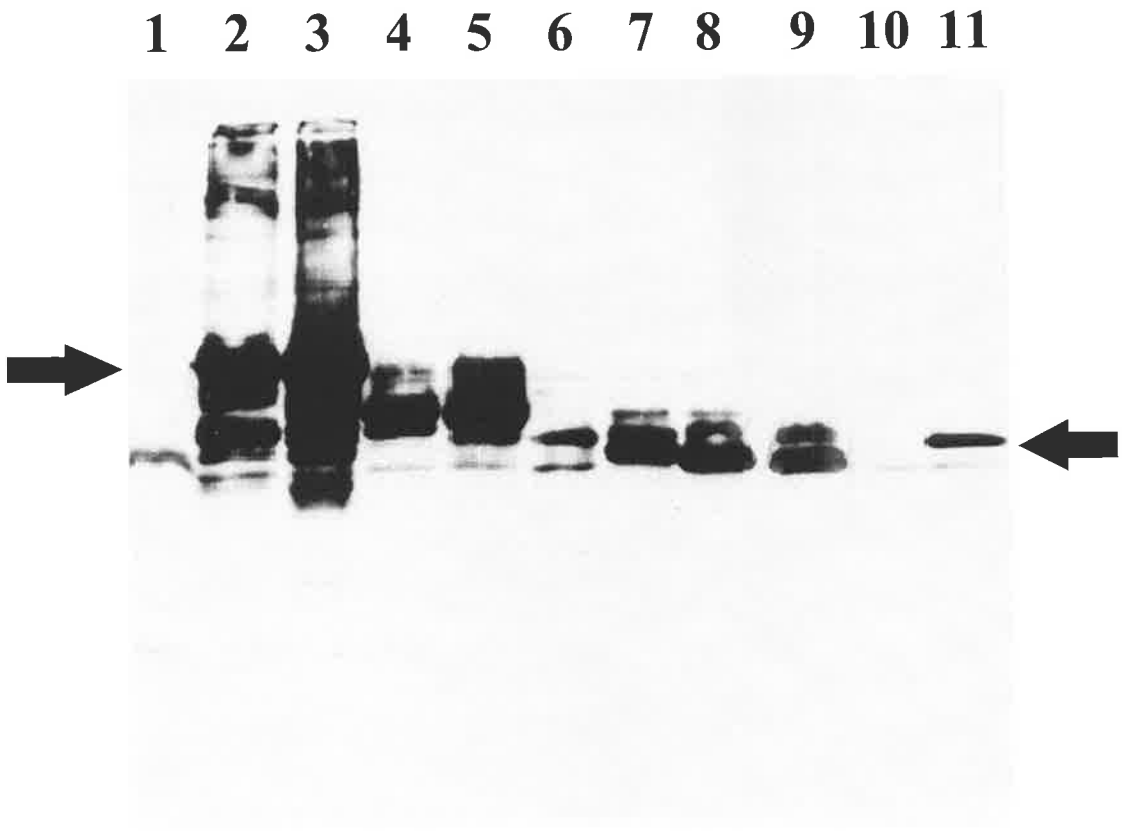


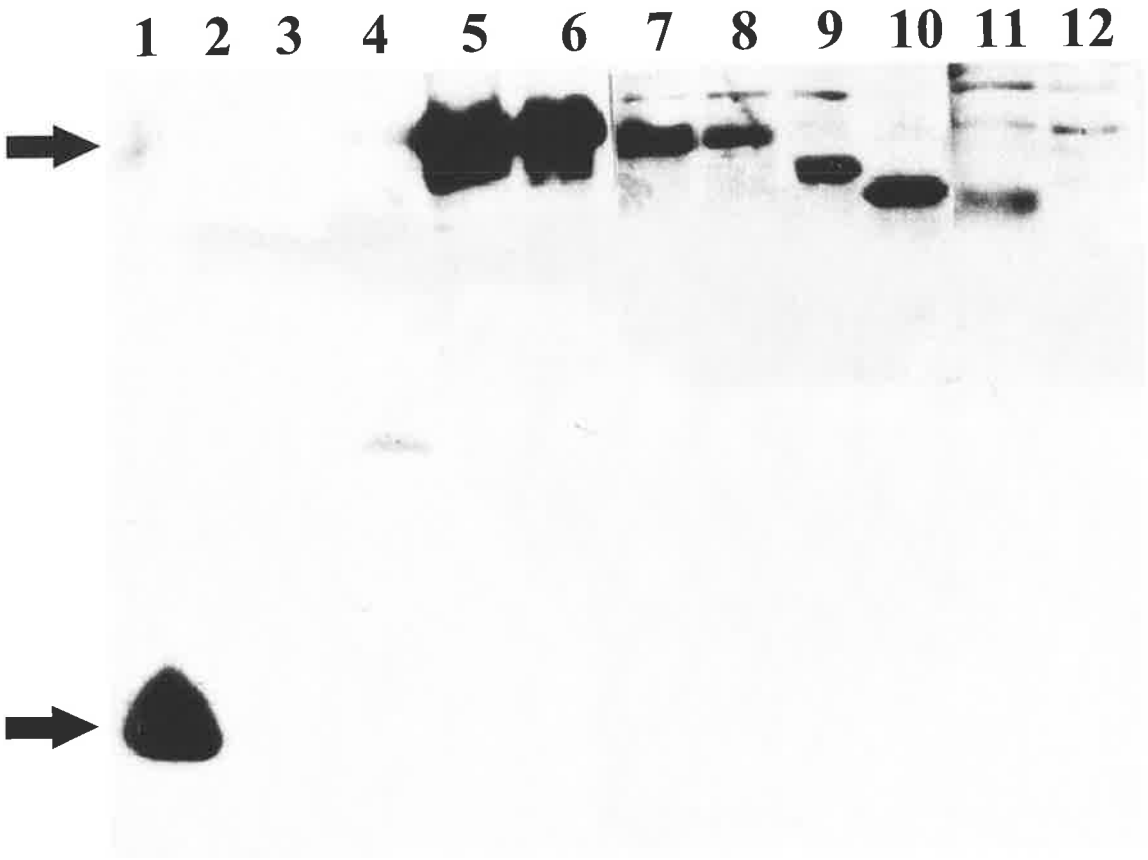
Fig. 5.3 Western blot analysis of the CstH::PhoA fusion proteins using antibody to alkaline phosphatase (AP).

CstH::PhoA fusion proteins generated by deletions into the C-terminal coding region of CstH were analysed using antibody to PhoA. Cell lysates were electrophoresed in SDS on 15% PAGE, transferred to a nitrocellulose filter and probed with the anti-alkaline phosphatase antibody and visualized by ECL detection. *E. coli* strains C75a and G206 were used as positive and negative controls, respectively. The arrows indicate the position of the largest CstH::PhoA fusion protein (left) and PhoA (right).

Lanes:

1. *E. coli* K-12 carrying pPM4502
2. *E. coli* K-12 carrying pPM4503
3. *E. coli* K-12 carrying pPM4504
4. *E. coli* K-12 carrying pPM4505
5. *E. coli* K-12 carrying pPM4506
6. *E. coli* K-12 carrying pPM4508
7. *E. coli* K-12 carrying pPM4507
8. *E. coli* K-12 carrying pPM4512
9. *E. coli* K-12 carrying pPM4513
10. *E. coli* strain G206 (- control)
11. *E. coli* strain C75a (+ control)


1 2 3 4 5 6 7 8 9 10 11


1 2 3 4 5 6 7 8 9 10 11 12

different end points across the mature CstH, and these have been used for expression and epitope analysis (Fig. 5.5).

5.2.2 N-terminal deletions in CstH

In order to define the precise location of the epitopes in CstH, deletion of the N-terminal coding region of CstH was performed. To make unidirectional deletions from the 5' end of *cstH* appropriate restriction sites were needed, in addition the hybrid gene required the *cstH* promoter and CstH signal sequence coding region, since it was assumed that the CstH::PhoA fusion proteins described above were being expressed from the *cstH* promoter. A plasmid having these characteristics was required. The DNA fragment in pPM4503 encoding an in-frame CstH::PhoA fusion protein from an earlier 3' deletion time point contains a nearly full-length *cstH* gene, and was chosen for this experiment.

5.2.2.1 Cloning of *cstH* promoter and signal sequence coding region

A 500 bp *Apa*I fragment of the *cst* operon from pPM484 containing the *cstH* promoter and signal sequence coding region was cloned into the *Apa*I site of pGEM-7Zf (Fig. 5.6). Transformants were screened for this fragment by white/blue colour selection on X-gal plates and restriction enzyme analysis. Some clones with the correct insertion were confirmed by sequencing. A clone with the *cstH* promoter and signal sequence coding region in the orientation of the T7 promoter was selected and designated pPM4528.

Fig. 5.5 Localization of C-terminal deletion endpoints within the CstH protein

The endpoints of the C-terminal deletions in CstH were defined by determining the sequence of the junction of the *cstH::phoA* fusions and defining the amount of residual *cstH* DNA. Numbers -1 to -22 indicate signal sequence. Amino acid numbers are corresponding to mature CstH. Arrows show the endpoints and these correspond to the appropriate plasmids as follows:

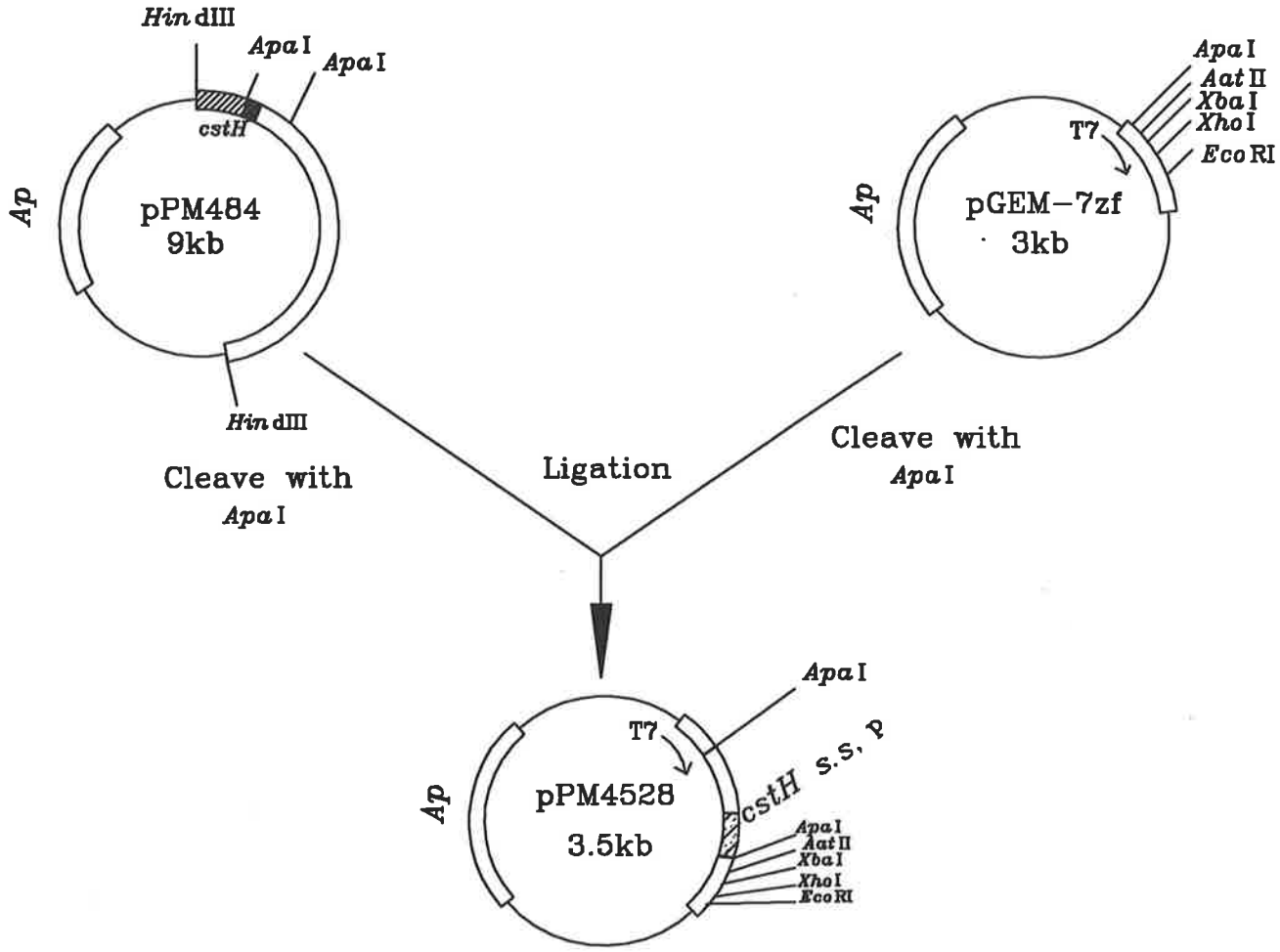
1. pPM4502
2. pPM4503
3. pPM4504
4. pPM4505
5. pPM4506
6. pPM4508
7. pPM4507
8. pPM4509
9. pPM4510
10. pPM4511
11. pPM4512
12. pPM4513
13. pPM4514

```
  1   CG TAT ACT GTT GGT CTT AAC GTA ACC AGT AAT GTT ATT TAA AGT GAA   47
                        -22
-22                      Met Leu Lys Ile Lys Tyr Leu Leu Ile Gly Leu   -12
 48  TGT ATG AGG GAT TCG ATG TTA AAA ATA AAA TAC TTA TTA ATA GGT CTT   95
                                        -1   1
-11  Ser Leu Ser Ala Met Ser Ser Tyr Ser Leu Ala Ala Ala Gly Pro Thr   5
 96  TCA CTG TCA GCT ATG AGT TCA TAC TCA CTA GCT GCA GCG GGG CCC ACT  143
    ↓13
  6  Leu Thr Lys Glu Leu Ala Leu Asn Val Leu Ser Pro Ala Ala Leu Asp   21
144  CTA ACC AAA GAA CTG GCA TTA AAT GTG CTT TCT CCT GCA GCT CTG GAT  191
          ↓12                    ↓11
 22  Ala Thr Trp Ala Pro Gln Asp Asn Leu Thr Leu Ser Asn Thr Gly Val   37
192  GCA ACT TGG GCT CCT CAG GAT AAT TTA ACA TTA TCC AAT ACT GGC GTT  239
                                    ↓10          ↓9
 38  Ser Asn Thr Leu Val Gly Val Leu Thr Leu Ser Asn Thr Ser Ile Asp   53
240  TCT AAT ACT TTG GTG GGT GTT TTG ACT CTT TCA AAT ACC AGT ATT GAT  287
    ↓8
 54  Thr Val Ser Ile Ala Ser Thr Asn Val Ser Asp Thr Ser Lys Asn Gly   69
288  ACA GTT AGC ATT GCG AGT ACA AAT GTT TCT GAT ACA TCT AAG AAT GGT  335
          ↓7                                                  ↓6
 70  Thr Val Thr Phe Ala His Glu Thr Asn Asn Ser Ala Ser Phe Ala Thr   85
336  ACA GTA ACT TTT GCA CAT GAG ACA AAT AAC TCT GCT AGC TTT GCC ACC  383
                                                          ↓5
 86  Thr Ile Ser Thr Asp Asn Ala Asn Ile Thr Leu Asp Lys Asn Ala Gly  101
384  ACC ATT TCA ACA GAT AAT GCC AAC ATT ACG TTG GAT AAA AAT GCT GGA  431
                                    ↓4
102  Asn Thr Ile Val Lys Thr Thr Asn Gly Ser Gln Leu Pro Thr Asn Leu  117
432  AAT ACG ATT GTT AAA ACT ACA AAT GGG AGT CAG TTG CCA ACT AAT TTA  479
                    ↓3
118  Pro Leu Lys Phe Ile Thr Thr Glu Gly Asn Glu His Leu Val Ser Gly  133
480  CCA CTT AAG TTT ATT ACC ACT GAA GGT AAC GAA CAT TTA GTT TCA GGT  527
                                        ↓2
134  Asn Tyr Arg Ala Asn Ile Thr Ile Thr Ser Thr Ile Lys ***          146
528  AAT TAC CGT GCA AAT ATA ACA ATT ACT TCG ACA ATT AAA TAA TTA TAT  575
                    ↓1
576  AAT AGA CGT AGC CTT CGA AAT AAA GGC TAC GTT GCT ATC TTT ATG TTT  623

624  GTG ATT TAT AGG CAT CAT TAA ATA GTC AAG CTT                      656
                                        HindIII
```

Fig. 5.6 Cloning of the *cstH* promoter and signal sequence coding region

Plasmid pPM484 was digested with *Apa*I and a 500bp fragment containing the *cstH* promoter and signal sequence coding region was cloned in *Apa*I site of plasmid pGEM-7zf. Transformants were selected by blue/white colour selection on X-gal plates. A plasmid containing this fragment was selected and designated pPM4528.


Hin dIII
Apa I
Apa I
cstH
Ap
pPM484
9kb
Hin dIII
Cleave with
Apa I
Ligation
Apa I
Aat II
Xba I
Xho I
Eco RI
T7
Ap
pGEM−7zf
3kb
Cleave with
Apa I
Apa I
T7
Ap
pPM4528
3.5kb
cstH
S.S. P
Apa I
Aat II
Xba I
Xho I
Eco RI

5.2.2.2 Cloning of a *cstH::phoA* fusion into pPM4528

A DNA fragment encoding the CstH::PhoA fusion protein from pPM4503, containing nearly full-length *cstH,* was cloned into plasmid pPM4528 downstream of the CstH signal sequence coding region. Transformants were screened on X-pho plates for blue colonies expressing AP activity which were analysed by restriction enzyme cleavage for detection of the inserted fragment. One blue colony with the correct cloned fragment was confirmed by sequencing, designated pPM4530 and used for deletion analysis. This clone contains appropriate restriction enzyme sites between the CstH::PhoA and CstH signal sequence coding fragments which allows unidirectional deletion into the 5' end of *cstH::phoA* and expression of fusion proteins via the CstH signal sequence (Fig. 5.7).

5.2.2.3 5' deletion *cstH*

Two restriction sites, *Aat*II and *Spe*I, were used for generating deletions. The *Spe*I site was used to generate a set of nested deletions into the CstH coding region from the 5' end of the *cstH* gene using Exonuclease III (ExoIII). The *Aat*II site was used as the protected site. The DNA from sequential time points was ligated and transformed into *E. coli* K-12 strain DH5α. Transformants were examined for AP activity on X-pho plates and blue colonies were screened for the size of the deletions by restriction enzyme analysis. A total of 15 different deletions were further characterized by sequencing and the length of *cstH* in fusions was defined. The clones were designated plasmids pPM4531-pPM4547 (Fig. 5.8).

5.2.3 Epitope mapping of CstH protein using C-terminal deletions of CstH

Initially, 2 Mabs against CS3, 10:2 and 11:2 were used for epitope analysis of CstH with 9 in-frame CstH::PhoA fusions derived by C-terminal deletion of CstH.

Fig. 5.7 Construction of plasmid pPM4530.

Plasmid pPM4503 was digested with *Xba*I and *Xho*I and the DNA fragment containing *cstH'::phoA'* was cloned between the *Xba*I and *Xho*I sites of pPM4528. Transformants were screened by restriction enzyme digestion and confirmed by sequencing. The correct construct was designated pPM4530.

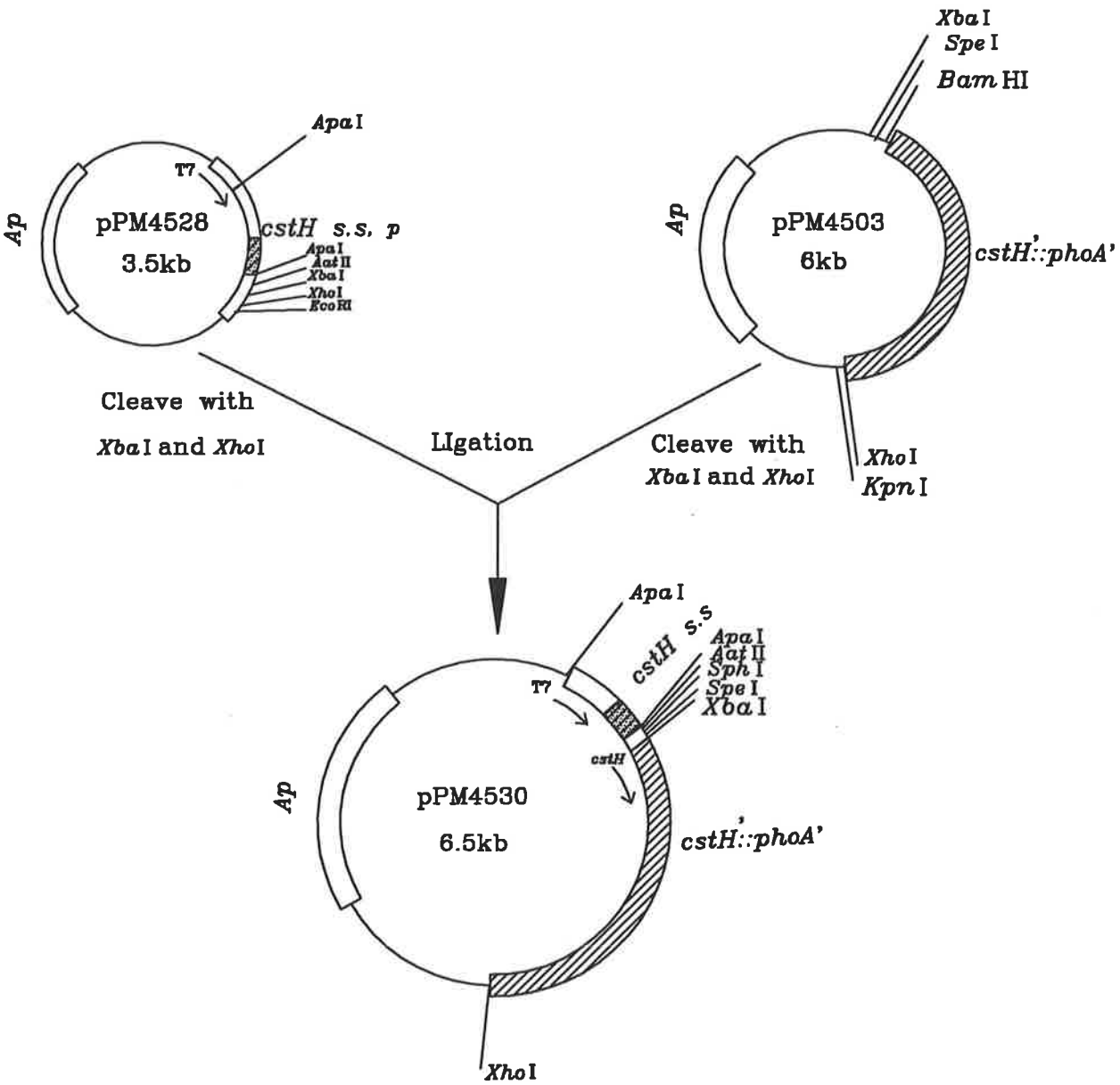

ApaI
T7
Ap
pPM4528
3.5kb
cstH s.s. p
ApaI
AatII
XbaI
XhoI
EcoRI
Cleave with
XbaI and XhoI
XbaI
SpeI
BamHI
Ap
pPM4503
6kb
cstH'::phoA'
XhoI
KpnI
LIgation
Cleave with
XbaI and XhoI
ApaI
cstH s.s
ApaI
AatII
SphI
SpeI
XbaI
T7
cstH
Ap
pPM4530
6.5kb
cstH'::phoA'
XhoI

Fig. 5.8 Localization of N-terminal deletion endpoints within the CstH protein

The endpoints of the N-terminal deletions in CstH were defined by determining the sequence of the junction of the *cstH::phoA* fusions and defining the amount of residual *cstH* DNA. Arrows show the endpoints of the truncated *cstH* fused to *phoA'* and the numbers correspond to the appropriate plasmids as follows. Mature CstH starts at #1 and -1 to -22 shows signal sequence.

1. pPM4531
2. pPM4532
3. pPM4533
4. pPM4534
5. pPM4536
6. pPM4537
7. pPM4539
8. pPM4540
9. pPM4541
10. pPM4538
11. pPM4542
12. pPM4543
13. pPM4545
14. pPM4546
15. pPM4547

```
  1   CG TAT ACT GTT GGT CTT AAC GTA ACC AGT AAT GTT ATT TAA AGT GAA   47

-22                      Met Leu Lys Ile Lys Tyr Leu Leu Ile Gly Leu   11
 48  TGT ATG AGG GAT TCG ATG TTA AAA ATA AAA TAC TTA TTA ATA GGT CTT   95
                                             -1   1
-11  Ser Leu Ser Ala Met Ser Ser Tyr Ser Leu Ala Ala Ala Gly Pro Thr   5
 96  TCA CTG TCA GCT ATG AGT TCA TAC TCA CTA GCT GCA GCG GGG CCC ACT  143
                                                 ↓1
 6   Leu Thr Lys Glu Leu Ala Leu Asn Val Leu Ser Pro Ala Ala Leu Asp   21
144  CTA ACC AAA GAA CTG GCA TTA AAT GTG CTT TCT CCT GCA GCT CTG GAT  191
                    ↓2                  ↓3
 22  Ala Thr Trp Ala Pro Gln Asp Asn Leu Thr Leu Ser Asn Thr Gly Val   37
192  GCA ACT TGG GCT CCT CAG GAT AAT TTA ACA TTA TCC AAT ACT GGC GTT  239
       ↓4                        ↓5
 38  Ser Asn Thr Leu Val Gly Val Leu Thr Leu Ser Asn Thr Ser Ile Asp   53
240  TCT AAT ACT TTG GTG GGT GTT TTG ACT CTT TCA AAT ACC AGT ATT GAT  287
     ↓6
 54  Thr Val Ser Ile Ala Ser Thr Asn Val Ser Asp Thr Ser Lys Asn Gly   69
288  ACA GTT AGC ATT GCG AGT ACA AAT GTT TCT GAT ACA TCT AAG AAT GGT  335
   ↓7                                  ↓8
 70  Thr Val Thr Phe Ala His Glu Thr Asn Asn Ser Ala Ser Phe Ala Thr   85
336  ACA GTA ACT TTT GCA CAT GAG ACA AAT AAC TCT GCT AGC TTT GCC ACC  383
   ↓9                                ↓10
 86  Thr Ile Ser Thr Asp Asn Ala Asn Ile Thr Leu Asp Lys Asn Ala Gly  101
384  ACC ATT TCA ACA GAT AAT GCC AAC ATT ACG TTG GAT AAA AAT GCT GGA  431
       ↓11              ↓12              ↓13                  ↓14
102  Asn Thr Ile Val Lys Thr Thr Asn Gly Ser Gln Leu Pro Thr Asn Leu  117
432  AAT ACG ATT GTT AAA ACT ACA AAT GGG AGT CAG TTG CCA ACT AAT TTA  479
                         ↓15
118  Pro Leu Lys Phe Ile Thr Thr Glu Gly Asn Glu His Leu Val Ser Gly  133
480  CCA CTT AAG TTT ATT ACC ACT GAA GGT AAC GAA CAT TTA GTT TCA GGT  527

134  Asn Tyr Arg Ala Asn Ile Thr Ile Thr Ser Thr Ile Lys ***          146
528  AAT TAC CGT GCA AAT ATA ACA ATT ACT TCG ACA ATT AAA TAA TTA TAT  575

576  AAT AGA CGT AGC CTT CGA AAT AAA GGC TAC GTT GCT ATC TTT ATG TTT  623

624  GTG ATT TAT AGG CAT CAT TAA ATA GTC AAG CTT                      656
```

Western blot analysis was performed using these Mabs with whole cell lysates of CFA grown bacteria harbouring plasmids encoding the CstH::PhoA fusion proteins.

Mab 11:2 did not react with fusions with deletions containing less than 122 amino acids of the mature CstH (Fig. 5.9). The largest non-reactive deletion contained 110 amino acids of mature CstH which indicated that part of the epitope for this Mab could be located between amino acids 111 and 122.

Mab 10:2 reacted with 8 fusions with as few as 28 amino acids of the mature CstH. The fusion in pPM4513 with only 23 amino acids could not be recognized (Fig. 5.10). This fusion also did not react with polyclonal antiserum against CS3. This result implies that at least part of a second epitope should be located between amino acids 23 and 28.

These results proved to be unreproducible and this was related to the level of expression of the fusion proteins, as will be seen in subsequent chapters. Consequently, it was difficult to obtain further data when more subclones of C-terminal CstH deletions were obtained and when the N-terminal deletions and the new Mabs were generated (Chapter 3). In all experiments using fusion proteins with polyclonal and monoclonal antibodies against CstH, only the fusions in pPM4503 and pPM4531 containing 144 amino acids of CstH (nearly full length CstH) could be reproducibly recognized in Western blot analysis (Fig. 5.11 and Fig. 5.12).

A number of attempts were made to overcome the possible problems related to expression of fusion proteins by using different growth media and conditions for the bacteria and by varying the preparation method of samples for Western blot analysis. However, no clear solution was obtained.

Fig. 5.9 Western blot analysis of the CstH::PhoA fusion proteins generated by deleting the C-terminus of CstH, using Mab 11:2.

CstH::PhoA fusion proteins generated by C-terminal deletion of CstH were analysed using Mab 11:2 to CS3. Cell lysates were electrophoresed in SDS on 15% PAGE, transferred to a nitrocellulose filter and probed with Mab 11:2 against CS3 and visualized by ECL detection. Wild type ETEC strain, PB176 and *E. coli* DH5α containing pPM4501 were used as positive and negative controls, respectively. The arrows indicate the position of the CstH (lower) and CstH::PhoA fusion proteins (upper).

Lanes:

1. PB176 (+control)
2. *E. coli* K-12 carrying pPM3500 containing *phoA'* genes (- control)
3. *E. coli* K-12 carrying pPM4502
4. *E. coli* K-12 carrying pPM4503
5. *E. coli* K-12 carrying pPM4504
6. *E. coli* K-12 carrying pPM4505
7. *E. coli* K-12 carrying pPM4506
8. *E. coli* K-12 carrying pPM4508
9. *E. coli* K-12 carrying pPM4507
10. *E. coli* K-12 carrying pPM4512
11. *E. coli* K-12 carrying pPM4513

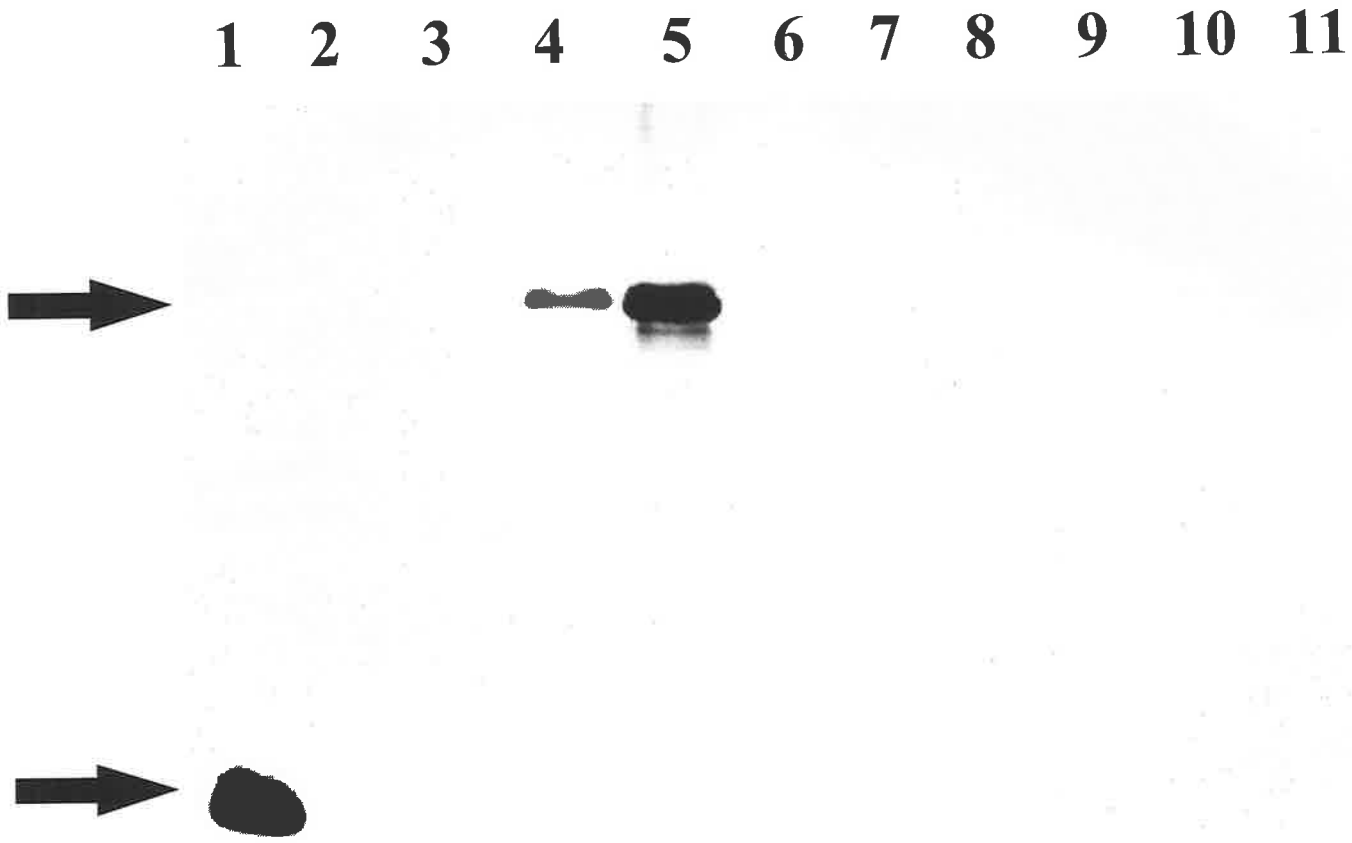

1 2 3 4 5 6 7 8 9 10 11

Fig 5.10 Western blot analysis of the CstH::PhoA fusion proteins generated by deletion of the C-terminus of CstH, using Mab 10:2

CstH::PhoA fusion proteins generated by C-terminal deletion of CstH were analysed using Mab 10:2 to CS3. Cell lysates were electrophoresed in SDS on 15% PAGE, transfered to a nitrocellulose filter and probed with Mab 10:2 against CS3 and visualized by ECL detection. Wild type ETEC strain, PB176 and *E. coli* DH5α containing pPM4501 were used as positive and negative controls, respectively. The arrows indicate the position of CstH (left) and CstH::PhoA fusion proteins (right).

Lanes:

1. PB176 (+control)
2. *E. coli* K-12 carrying pPM3500 containing *phoA'* genes (- control)
3. *E. coli* K-12 carrying pPM4502
4. *E. coli* K-12 carrying pPM4503
5. *E. coli* K-12 carrying pPM4504
6. *E. coli* K-12 carrying pPM4505
7. *E. coli* K-12 carrying pPM4506
8. *E. coli* K-12 carrying pPM4508
9. *E. coli* K-12 carrying pPM4507
10. *E. coli* K-12 carrying pPM4512
11. *E. coli* K-12 carrying pPM4513

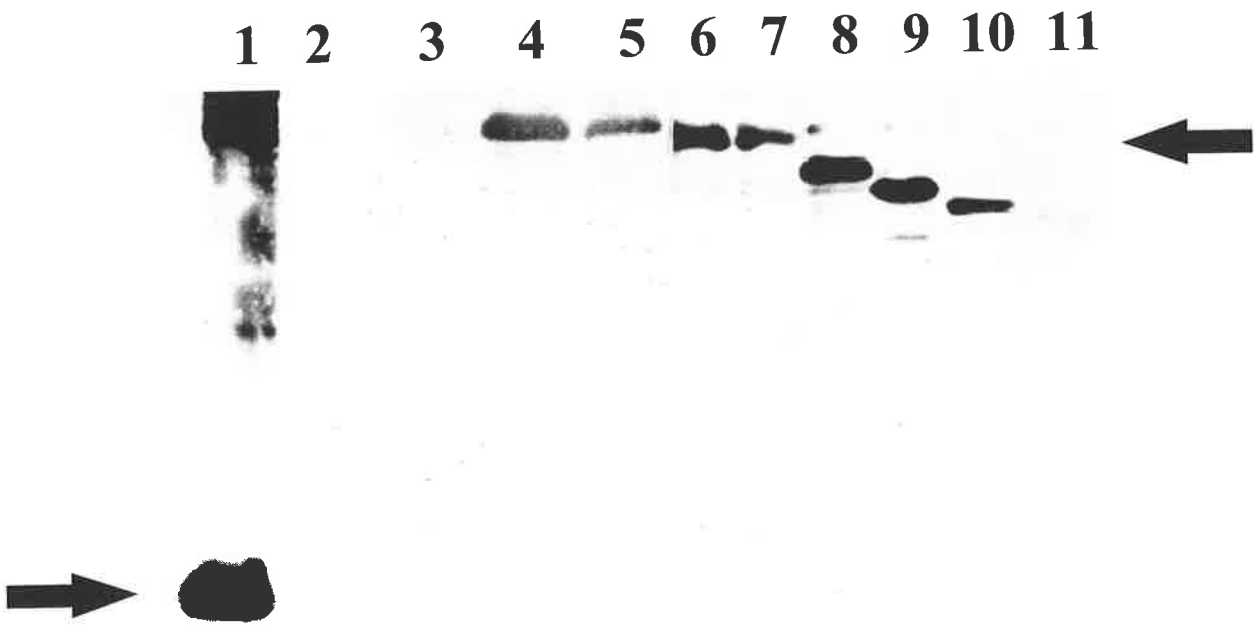

1 2 3 4 5 6 7 8 9 10 11

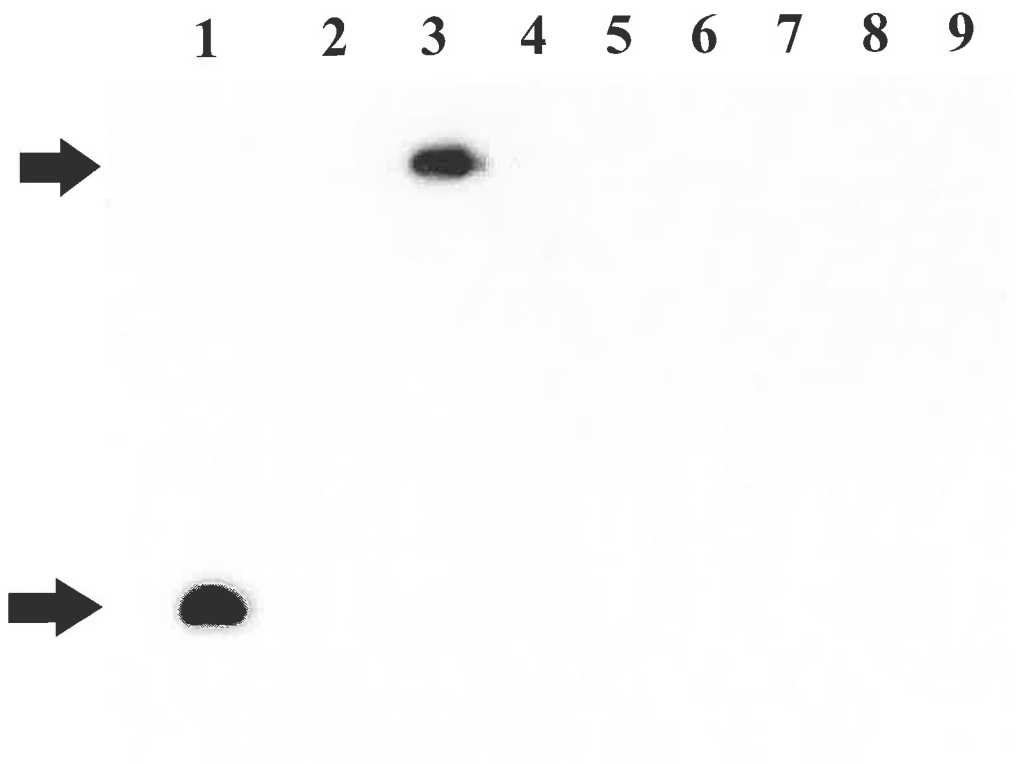
Fig 5.11 Western blot analysis of the CstH::PhoA fusion proteins generated by deletion of the C-terminus of CstH

CstH::PhoA fusion proteins generated by C-terminal deletion of CstH were analysed using polyclonal antibody to CS3. The antiserum was absorbed with *E. coli* K-12 strain DH5α containing pBluescript SK. Cell lysates were electrophoresed in SDS on 15% PAGE, transferred to a nitrocellulose filter and probed with the antiserum and visualized by ECL detection. Purified CS3 and *E. coli* containing pPM4501 were used as positive and negative controls, respectively. The arrows indicate the position of the CstH (lower) and CstH::PhoA fusion proteins (upper).

Lanes:

1. Purified CS3 (+control)
2. *E. coli* K-12 strain DH5α carrying pPM3500 containing *phoA'* genes (- control)
3. *E. coli* K-12 strain DH5α carrying pPM4503
4. *E. coli* K-12 strain DH5α carrying pPM4504
5. *E. coli* K-12 strain DH5α carrying pPM4505
6. *E. coli* K-12 strain DH5α carrying pPM4506
7. *E. coli* K-12 strain DH5α carrying pPM4508
8. *E. coli* K-12 strain DH5α carrying pPM4507
9. *E. coli* K-12 strain DH5α carrying pPM4512

The other deletions were not used in this experiment


1 2 3 4 5 6 7 8 9


1 2 3 4 5 6 7 8 9 10 11 12

5.3 Discussion

In this chapter an epitope analysis of CstH was attempted based on CstH::PhoA fusion proteins in which varying amounts of either the N- or C-terminus of CstH had been deleted.

Two epitopes were identified in the initial experiments using two Mabs (10:2 and 11:2) and 9 fusion proteins based on CstH C-terminal deletions. It was concluded that the epitopes should be located before amino acids 23 and 122 of the mature CstH. It was not possible to extend this mapping to the 19 Mabs isolated in chapter 3 and so define the precise location of the epitopes. Since only the fusion protein with 3 amino acids deleted from the C-terminus reliably reacted with the Mabs and polyclonal antisera to CS3 in subsequent experiments.

Deletion mutagenesis has been used for mapping of a continuous epitope on the baculovirus capsid protein, and it was concluded that this technique could be used as an alternative to the other procedures for mapping of linear epitopes, such as peptide synthesis (PEPSCAN) (Gross and Rohrmann, 1990). PEPSCAN failed to map the epitopes of K88 fimbriae (van Zijderveld et al., 1990) and it was concluded that its epitopes are probably conformational. Antibodies raised against synthetic peptides mimicking predicted epitopes of the major subunit of K88 also could not recognize intact fimbriae (Krogfelt, 1987) again supporting the notion that the epitopes of K88 are conformational.

It seems that, like K88, the epitopes of CstH or at least the epitopes recognized by the Mabs used are conformational and dependent upon the quaternary structure of protein formed by intact CstH. A conformational epitope for Mab 11:2 on the native CS3 pili has been predicted (Chapter 4). Thus deletion of more than the terminal 3 amino acids of the mature CstH protein appears to disturb the structure of the whole

protein and the truncated proteins are unable to be recognized by antibodies. Recognition of CstH by Mabs on Western blot that was reported in Chapter 3 and 4 may have been due to some renaturation of CstH during the experiment (van Dam,1994). There is also the possibility that the deletion into CstH destabilizes the protein or makes it particularly sensitive to the denaturing conditions, such that during sample preparation and electrophoresis, the fusion proteins or at least the CstH domains are denatured or degraded and no longer able to react with the antibodies.

It is feasible that the initial result (Fig 5.4) could be due to the use of large amounts of the fusion proteins, part of which remained undenatured and therefore could still react with the antibodies.

Hybrid major subunits of K88 have been constructed in which various parts of the $K88_{ab}$ and $K88_{ac}$ major subunits were exchanged, or non-conserved amino acid residues were substituted by mutagenesis. These proteins were used to study the antigenicity and receptor binding, and several regions or amino acids involved in the serotype-specific epitopes and receptor binding sites were identified (Bakker *et al.*, 1992).

The results obtained from the epitope analysis of CstH and its antigenic variation (Chapter 4) support the conclusions made with K88 that the antigenic determinants of these pili are conformational and that a more suitable procedure for epitope mapping and receptor binding site analysis may be insertion mutagenesis. This can be accomplished either randomly, or in certain regions that have been predicted by computer analysis to represent exposed domains of the protein. This approach has been taken in the following chapters.

Chapter 6

Insertion of heterologous peptide into CstH

6.1 Introduction

The immunogenicity of poor antigens can be increased by coupling them to carriers such as bovine serum albumin and tetanus toxoid. Recent studies have shown that the immunogenicity of a poor antigen can also be greatly enhanced when it is genetically fused to bacterial components and expressed in an appropriate host strain (Clements, 1990).

Many cell surface structures such as outer membrane proteins: LamB, PhoE, OmpA and TraT (Agterberg *et al.*,1990; Hayes *et al.*, 1991; Ruppert *et al.*, 1994; Taylor *et al.*, 1990), flagella and fimbriae including: P fimbriae, K88, type 1 and type 4 fimbriae (Bakker *et al.*, 1990; Bouquset *et al.*, 1994., Van Die *et al.*, 1990) have been used for expression of antigenic determinants. Immunization with these recombinant molecules can lead to an immune response against both the carrier and relevant determinants when injected into animals. This approach is considered to be useful for the expression of critical antigenic determinants of pathogens for use in vaccine development.

In this study the CS3 fibrillar adhesin system has been used for the development of a new system for expression of foreign antigenic determinants. CS3 fimbriae have been relatively well studied. The genes encoding biosynthesis of the CS3 pili have been cloned (Manning *et al.*, 1985), sequenced and characterized (Jalajakumari *et al.*, 1989). The organization of the genes involved in the biosynthesis and expression of the CS3 is shown in Fig 1.3.

The CS3 system has some potential advantages and may be a good candidate for expression of foreign epitopes for vaccine development. These advantages include:

1. CS3 is a good immunogen (Reid *et al.*, 1993), which may be important for the stimulation of an immune response directed against the inserted epitope.

2. CS3 is a colonization factor (Wenneras et al, 1990) and consequently a hybrid protein may possess both antigenic and adhesive properties which are important for live oral vaccines.

3. The inserted antigen will be transported to and expressed on the cell surface of the bacteria in large amounts.

4. CS3 pili are thin (~2nm), flexible, wiry fibrillar (Levine *et al.*, 1984). CS3 pili are thinner and more flexible than most of the fimbriae from ETEC strains (de Graaf and Gaastra, 1994). They may expose a larger proportion of subunit protein on the surface rather than it being hidden within the pilus structure or in pilin-pilin contact regions, when compared to flagella and other pili which are much thicker structures. Consequently, it seems likely that the foreign epitope will be exposed on the surface of the pili.

5. The CstH protein contains many proline and glycine residues (Jalajakumari *et al.*, 1989) which can induce turns in the secondary structure of the protein (Sutcliffe *et al.*, 1994) providing areas that could be exposed on the protein. Foreign antigenic determinants inserted in these regions would be expected to be exposed on the surface of the protein.

Enterotoxigenic *E. coli* strains produce a heat-labile enterotoxin (LT) which is structurally and immunologically related to cholera toxin (CT). The holotoxin consists of an A subunit and a pentamer of B subunits. LT toxin and its B subunit are strong immunogens and can stimulate production of serum and secretory antibodies when

delivered orally. This property, as well as its high stability, capacity to bind to receptors on the target cell, and its existence in ETEC of different serotypes and colonization factor types, have made this protein a good candidate for the development of the oral vaccines against LT^+ ETEC (Nashar *et al.,* 1993; Spangler, 1992).

There have been several efforts to develop a vaccine for cholera and ETEC based on CT-B and LT-B. Effective immunity has been obtained using a vaccine containing both whole-cell and toxoid antigen (Ahren *et al.,* 1993; Jertborn *et al.,*1994).

Synthetic peptides corresponding to different regions of CT-B (which are conserved in hLT and pLT) have been used for immunization and the generation of antibodies. Antibodies directed against residues 50-64 of CT-B cross-reacted with and neutralized hLT and pLT (Jacob *et al.,*1984). Expression of this region in *Salmonella* flagella and their use for immunization resulted in antibodies that recognized CT-B in a solid-phase assay (Newton *et al.,* 1989).

In this chapter an epitope of hLT-B consisting of residues 44-64, which are conserved in CT-B and LT-B, has been used for insertion into CstH to evaluate the potential of CS3 as a delivery system for heterologous antigenic determinants.

6.2 Results

The restriction sites available for the insertion of foreign epitopes into CS3 are somewhat limited and their use is complicated if the entire biosynthesis region is used in genetic manipulations. But the genetic organisation of the *cst* gene cluster (see Fig 1.3) makes it feasible to work with a much smaller fragment containing only the *cstH* gene. A second cassette containing all of the assembly genes could be cloned separately, and these functions could be supplied in trans.

6.2.1.1 Cloning of the *cstH* gene cassette

Plasmid pPM484 was digested with *Sna*BI and *Hin*dIII and a 650bp fragment containing the CS3 subunit gene (*cstH*) including its promoter and signal sequence was cloned into pBluescriptSK which had been digested with *Hin*dIII and *Sma*I (Fig. 6.1). Transformants were screened for *cstH* using blue/white colour selection on X-gal plates followed by restriction enzyme analysis. One clone, designated pPM4555, containing a fragment of the correct size was characterized further by DNA sequencing and used in subsequent experiments.

6.2.1.2 Cloning of the CS3 assembly genes cassette

In order to construct the CS3 assembly gene cassette, the 4200 bp *Hin*dIII-*Dra*II fragment of the *cst* region from pPM484 was cloned between the *Hin*dIII and *Dra*II sites of plasmid pACYC184 (Fig. 6.2). This clone, which contains all of the genes for fimbrial biogenesis except for the major structural subunit, was designated pPM4551 and used for expression of mutant CS3 subunits (CstH) and hybrid proteins. This allows hybrid/mutant CstH proteins to be supplied in trans and assembled.

6.2.2 Insertion of foreign epitopes into the CS3 subunit

6.2.2.1 Mutagenesis in *cstH*

The exposed (most likely epitopes) and hypervariable regions of a protein are good candidates for insertion and expression of heterologous peptides. These areas can be defined by experimental approaches such as epitope mapping, or from the predicted secondary structure of the protein. Since no variable sequence domains could be found in CstH and epitope analysis of the protein was not entirely successful, it was decided to

Fig. 6-1 Construction of a *cstH* cassette

The *cstH* gene was separated from the remainder of the *cst* operon by cloning of the *Hin*dIII-*Sna*BI fragment of pPM484 between the *Hin*dIII and *Sma*I sites of pBluescript SK. Transformants were selected by blue/white colour selection on X-gal plates and by Ap^R and confirmed by sequencing. The plasmid generated was designated pPM4555.

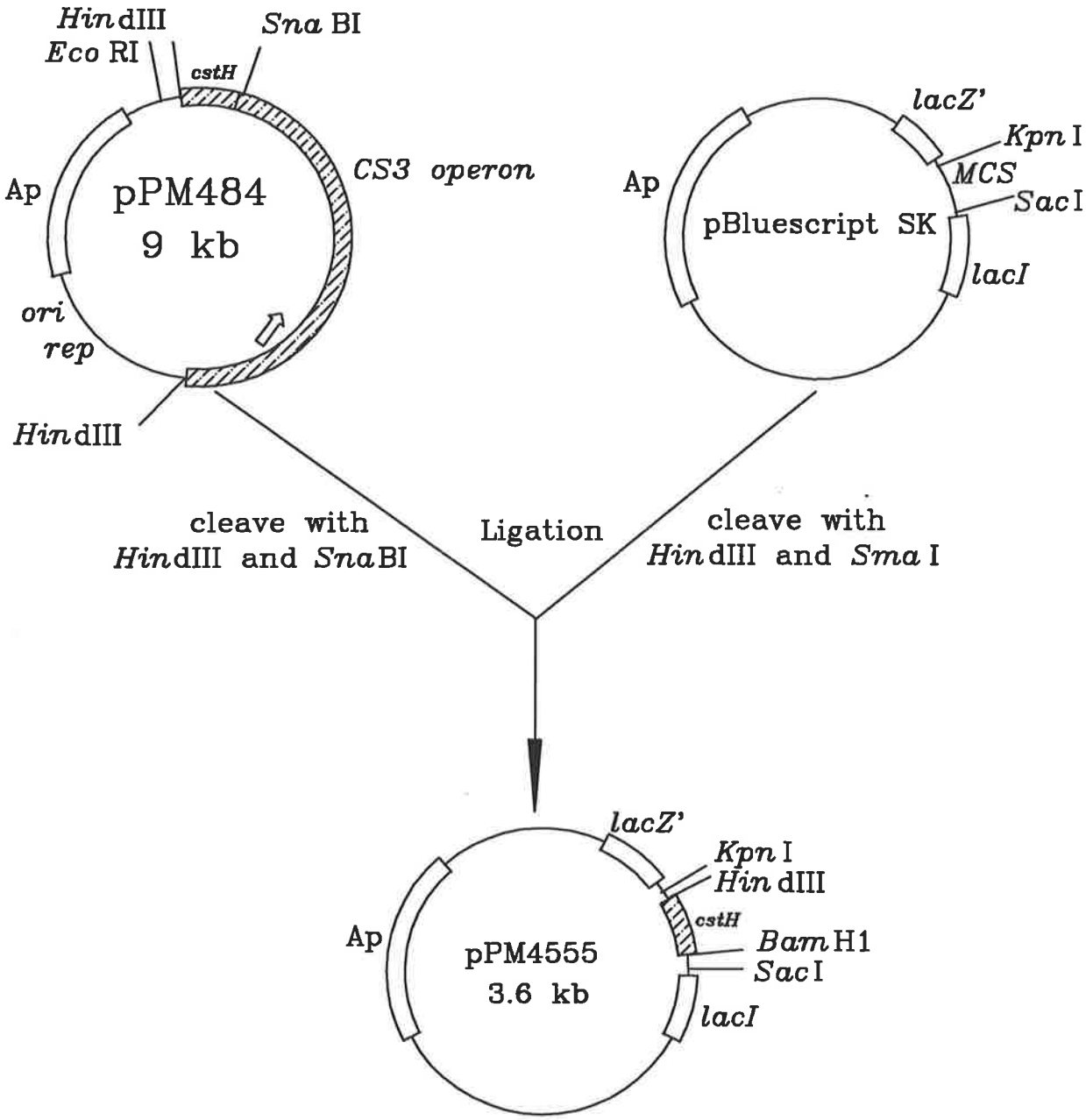
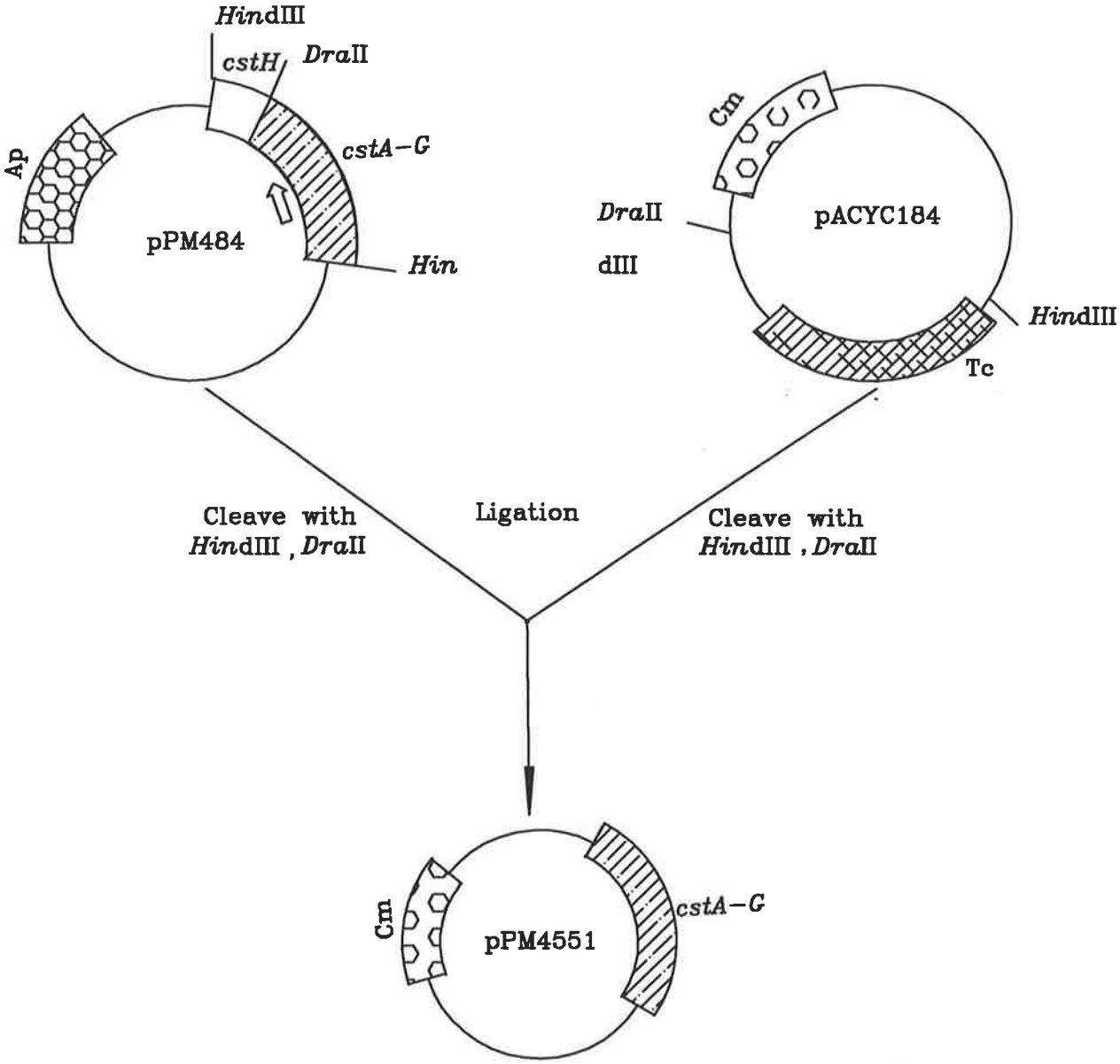

HindIII
Eco RI
SnaBI
cstH
Ap
pPM484
9 kb
CS3 operon
ori
rep
HindIII
lacZ'
KpnI
MCS
SacI
Ap
pBluescript SK
lacI
cleave with
HindIII and SnaBI
Ligation
cleave with
HindIII and SmaI
lacZ'
KpnI
HindIII
cstH
Ap
pPM4555
3.6 kb
BamH1
SacI
lacI

Fig. 6-2 Construction of a CS3 assembly cassette.

The genes encoding the assembly proteins of the *cst* operon (*cstA-G*) were cloned. Plasmid pPM484 was digested with *Hin*dIII and *Dra*II and the DNA fragment was ligated between the *Hin*dIII and *Dra*II sites of pACYC184. Transformants were selected by Cm^R and screened for Tc^S due to the replacement of the Tc gene by the *cstA-G* genes. The plasmid generated was designated as pPM4551.


HindIII
cstH
DraII
cstA–G
Ap
pPM484
Hin
Cm
DraII
dIII
pACYC184
HindIII
Tc
Cleave with
HindIII , DraII
Ligation
Cleave with
HindIII , DraII
Cm
pPM4551
cstA–G

use the computer programs to predict the exposed regions of CstH for insertion of foreign peptides.

Three regions, including amino acids 65-68, 98-100 and 108-110 of the mature protein, which have been predicted to be surface exposed areas of CstH were considered. This prediction was based on the proposition that the exposed regions were located in or adjacent to turns in the secondary structure of the protein as predicted by a Chou and Fasman (1987) analysis (Fig. 6.3). Analysis of the secondary structure-solvent accessibility of CstH by PHD (Profile fed neural network systems from HeiDelberg) program (Rost and Sander, 1993, 1994) and PROFILEGRAPH program (Hofmann and Stoffe, 1989) supported this prediction and defined these areas as loops and exposed regions of the protein (Fig 6.4a and 6.4b). A summary of the location of the predicted epitopes within CstH is shown in Fig. 6.5.

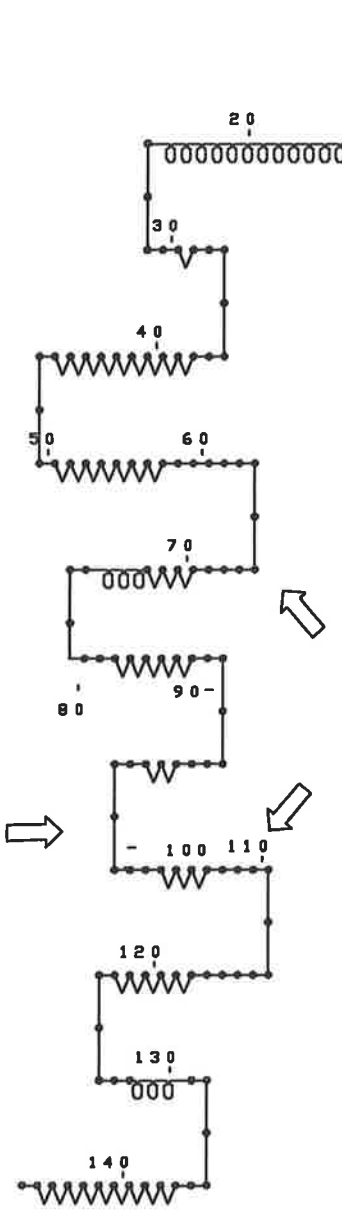
In order to introduce suitable cleavage sites into three regions of *cstH,* PCR site-directed mutagenesis was used. Three mutant oligos containing an *Xba*I site (for mutation of nt 4413-4417) or *Sty*I site (for mutation of nt 4515-4518 and 4542-4545) were used in the mutagenesis of *cstH.* The PCR products were cloned into pBluescript SK or pGEM3zf and the transformants were screened for the *Xba*I or *Sty*I sites by restriction enzyme analysis and confirmed by DNA sequencing. The clones with the desired mutation and correct sequence for the rest of the *cstH* gene were selected (pPM4556, pPM4557, pPM4559, respectively) and used for insertion of the foreign epitopes. Mutagenesis in *cstH* resulted in the substitution of amino acids #66 and 67 ($Ser_{66}\rightarrow$Leu and $Lys_{67}\rightarrow$Glu), amino acid #100 ($Ala_{100}\rightarrow$Leu) and #109 ($Asn_{109}\rightarrow$Leu) of the mature CstH protein in the three mutants, respectively.

Fig. 6.3 Predicted secondary structure of the CstH protein

The secondary structure of CstH was predicted using the algorithm of Chou and Fasman (1978). The mature CstH protein is 146 amino acids in length. The arrows show the predicted exposed sites of the protein.

Secondary Structure Prediction

FILE:CS3.AMI SIZE: 146aa SEQ.: 1- 146 FUNCTION:Chou and Fasman



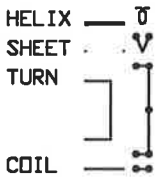


Fig 6.4 Secondary structure analysis of the amino acid sequence of the mature CstH protein.

a:

The secondary structure and solvent accessibility of the mature CstH protein was determined using the PHD program (Rost and Sander, 1993, 1994). The underlined, bold sequences correspond to the predicted epitopes.

Abbreviations:
PHDsec PHD: Profile network prediction HeiDelberg
AA : amino acid sequence
secondary structure:
HEL: H=helix, E=extended (sheet), blank=other (loop)
Rel: Reliability index of prediction (0-9) prH: 'probability' for assigning helix
prE: 'probability' for assigning strand prL: 'probability' for assigning loop
note: the 'probabilities' are scaled to the interval 0-9, e.g.,
prH=5 means, that the first output node is 0.5-0.6
SUB: a subset of the prediction, for all residues with an expected average accuracy > 82%
note: for this subset the following symbols are used:
L: is loop (for which above " " is used)
".": means that no prediction is made for this residue, as the reliability is: Rel < 5

Abbreviations:
PHDacc solvent accessibility:
3st: relative solvent accessibility (acc) in 3 states: b = 0-9%, i = 9-36%, e = 36-100%.
Rel: Reliability index of prediction (0-9)
P_3: predicted relative accessibility in 3 states
note: for convenience a blank is used intermediate (i).
10st:relative accessibility in 10 states:= n corresponds to a relative acc. of n*n %
SUB: a subset of the prediction, for all residues with an expected average correlation > 0.69
note: for this subset the following symbols are used:
"I": is intermediate (for which above " " is used)
".": means that no prediction is made for this residue, as the reliability is: Rel < 4

b:

The hydropathy and accessible surface area of the mature CstH protein were determined using prograph program according to the different algorithms in the PROFILEGRAPH. X and Y axes indicate the sequence position and hydrophilicity values respectively.

a

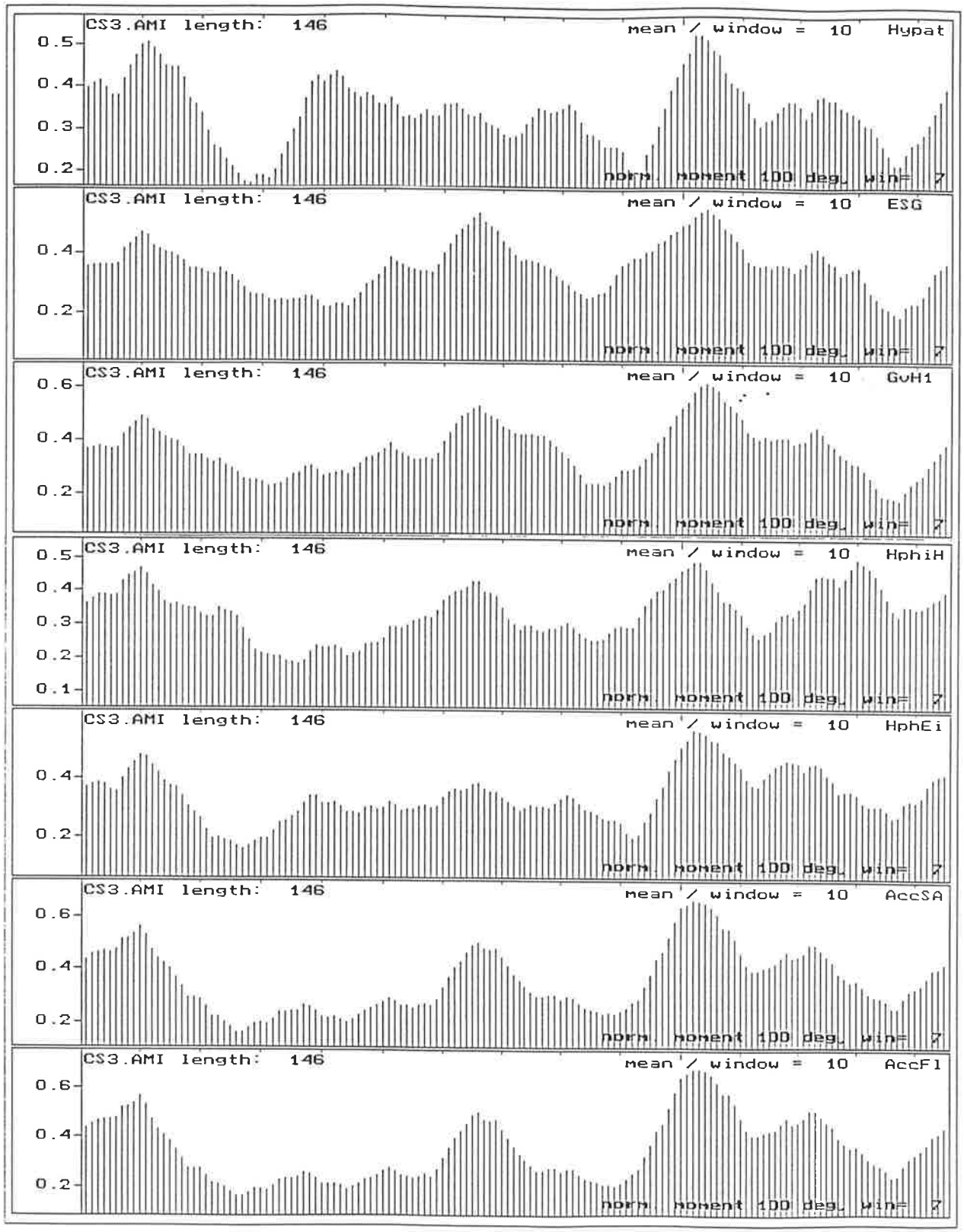
Prediction of: -secondary structure-solvent accessibility of CstH

```
                 ....,....10...,....20...,....30...,....40...,....50...,....60
         AA      |AAGPTLTKELALNVLSPAALDATWAPQDNLTLSNTGVSNTLVGVLTLSNTSIDTVSIAST|
         PHD sec |      HHHHHHHH   HHHH         EEE   EEEEEEEEEEEE     EEEEEEE|
         Rel sec |999842137775213122331144556541685366153688999993276526999985|
         prH sec |000023347776543434554422111211000000000000000000000000000000|
         prE sec |000012321000010011111112110024786322465788999986412247999887|
         prL sec |999864321112336453233455667664112677423110000003577752000012|
subset:  SUB sec |LLLL....HHHH............LLLL..EEE.LL.E.EEEEEEEE..LLL.EEEEEEE|
ACCESSIBILITY
3st:     P_3 acc |eeeeebbeebbbebbbbbbbebebbeeeebebeeeebbbbbbbbbbbbbeebbebbbbbbe|
10st:    PHD acc |997770076000700000007070067770606767000000000007600600000007|
         Rel acc |953553152474355302425430214431231313310246576140311213835532|
subset:  SUB acc |ee.ee..e.bbb.bb...b.eb....ee............bbbbb.b.......b.bb..|

                 ....,....70...,....80...,....90...,....100..,....110..,....120
         AA      |NVSDTSKNGTVTFAHETNNSASFATTISTDNANITLDKNAGNTIVKTTNGSQLPTNLPLK|
         PHD sec |E        EEEEEEE     EEEEEEE    EEEEE     EEEEE           EE|
         Rel sec |136689998689986347985144898537636856416897799923897679887627|
         prH sec |000000000000000000000000000000000000000010000000000000000000|
         prE sec |531100001789987521012466888631137877641001899953101210011257|
         prL sec |467789898110012368986423100257752122257898100046888788887741|
subset:  SUB sec |..LLLLLLEEEEEE..LLLL...EEEE.LL.EEEE..LLLLEEEE..LLLLLLLLLL.E|
ACCESSIBILITY
3st:     P_3 acc |ebeeebeebebbbbbeeeeeebbbbbbbb eebebebeeebeebbbbeb eeeebbeebebe|
10st:    PHD acc |708770980700006777670000000057606060677096000703977600970606|
         Rel acc |423530761352641423134036123014051424155142064510434210440133|
subset:  SUB acc |e..e..ee..b.bb.e....b..b.....e.b.b.b.ee.e..bbe..e.e...ee....|

                 ....,....130..,....140..,146
         AA      |FITTEGNEHLVSGNYRANITITSTIK|
         PHD sec |EEE   EEEEEE   EEEEEEEEEE |
         Rel sec |896466279982112678788767 49|
         prH sec |000000000000000000000000000|
         prE sec |887321588885444788888776 0|
         prL sec |102677410014555211111121 39|
subset:  SUB sec |EEE.LL.EEEE....EEEEEEEEE.L|
ACCESSIBILITY
3st:     P_3 acc |bbbeebee bbbee  bbbeb bebe|
10st:    PHD acc |000670674000763500060506 09|
         Rel acc |342161160550301151618122 17|
subset:  SUB acc |.b..e..e.bb.....b.b.b....e|
```

b


CS3.AMI length: 146
mean / window = 10 Hypat
0.5
0.4
0.3
0.2
norm. moment 100 deg. win=
CS3.AMI length: 146
mean / window = 10 ESG
0.4
0.2
norm. moment 100 deg. win=
CS3.AMI length: 146
mean / window = 10 GvH1
0.6
0.4
0.2
norm. moment 100 deg. win=
CS3.AMI length: 146
mean / window = 10 HphiH
0.5
0.4
0.3
0.2
0.1
norm. moment 100 deg. win=
CS3.AMI length: 146
mean / window = 10 HphEi
0.4
0.2
norm. moment 100 deg. win=
CS3.AMI length: 146
mean / window = 10 AccSA
0.6
0.4
0.2
norm. moment 100 deg. win=
CS3.AMI length: 146
mean / window = 10 AccFl
0.6
0.4
0.2
norm. moment 100 deg. win=

A

B

C

D

E

F

G

Fig. 6.5 Localization of the predicted epitopes within CstH.

Nucleotide and amino acid sequences of *cstH* and CstH are presented. Underlined, *italic* sequences indicate the predicted epitopes within CstH which were chosen for insertion of heterologous epitopes.

Localization of predicted surface-exposed epitopes within CstH

```
  1   CG TAT ACT GTT GGT CTT AAC GTA ACC AGT AAT GTT ATT TAA AGT GAA   47

 -1                      Met Leu Lys Ile Lys Tyr Leu Leu Ile Gly Leu  -12
 48  TGT ATG AGG GAT TCG ATG TTA AAA ATA AAA TAC TTA TTA ATA GGT CTT   95
                                             -1   1
-11  Ser Leu Ser Ala Met Ser Ser Tyr Ser Leu Ala Ala Ala Gly Pro Thr   5
 96  TCA CTG TCA GCT ATG AGT TCA TAC TCA CTA GCT GCA GCG GGG CCC ACT  143

  6  Leu Thr Lys Glu Leu Ala Leu Asn Val Leu Ser Pro Ala Ala Leu Asp   21
144  CTA ACC AAA GAA CTG GCA TTA AAT GTG CTT TCT CCT GCA GCT CTG GAT  191

 22  Ala Thr Trp Ala Pro Gln Asp Asn Leu Thr Leu Ser Asn Thr Gly Val   37
192  GCA ACT TGG GCT CCT CAG GAT AAT TTA ACA TTA TCC AAT ACT GGC GTT  239

 38  Ser Asn Thr Leu Val Gly Val Leu Thr Leu Ser Asn Thr Ser Ile Asp   53
240  TCT AAT ACT TTG GTG GGT GTT TTG ACT CTT TCA AAT ACC AGT ATT GAT  287

 54  Thr Val Ser Ile Ala Ser Thr Asn Val Ser Asp Thr Ser Lys Asn Gly   69
288  ACA GTT AGC ATT GCG AGT ACA AAT GTT TCT GAT ACA TCT AAG AAT GGT  335

 70  Thr Val Thr Phe Ala His Glu Thr Asn Asn Ser Ala Ser Phe Ala Thr   85
336  ACA GTA ACT TTT GCA CAT GAG ACA AAT AAC TCT GCT AGC TTT GCC ACC  383

 86  Thr Ile Ser Thr Asp Asn Ala Asn Ile Thr Leu Asp Lys Asn Ala Gly  101
384  ACC ATT TCA ACA GAT AAT GCC AAC ATT ACG TTG GAT AAA AAT GCT GGA  431

102  Asn Thr Ile Val Lys Thr Thr Asn Gly Ser Gln Leu Pro Thr Asn Leu  117
432  AAT ACG ATT GTT AAA ACT ACA AAT GGG AGT CAG TTG CCA ACT AAT TTA  479

118  Pro Leu Lys Phe Ile Thr Thr Glu Gly Asn Glu His Leu Val Ser Gly  133
480  CCA CTT AAG TTT ATT ACC ACT GAA GGT AAC GAA CAT TTA GTT TCA GGT  527

134  Asn Tyr Arg Ala Asn Ile Thr Ile Thr Ser Thr Ile Lys ***          146
528  AAT TAC CGT GCA AAT ATA ACA ATT ACT TCG ACA ATT AAA TAA TTA TAT  575

576  AAT AGA CGT AGC CTT CGA AAT AAA GGC TAC GTT GCT ATC TTT ATG TTT  623

624  GTG ATT TAT AGG CAT CAT TAA ATA GTC AAG CTT                      656
                                        HindIII
```

6.2.2.2 Expression of mutant CstH

The effect of the mutations on the stability of the mutant CstH proteins was determined by Western blot analysis. Plasmids pPM4555, pPM4556, pPM4557, pPM4559 were transformed into the *E. coli* K-12 strain DH5α to give strains E2755, E2756, E2757 and E2759, respectively. The *cstH* gene in these plasmids has its own promoter. The cells harbouring these plasmids were grown on CFA agar and analysed by Western blot using a polyclonal antibody against purified native CS3. This revealed a band corresponding to the CS3 subunit from the wild type ETEC strain, PB176 (Fig. 6.6). The same results were obtained using two anti-CS3 MAbs 10:2 and 11:2. These results suggest that the mutations did not affect the stability or overall antigenic structure of the protein (Fig 6.7).

6.2.3 Cloning of the *eltB* gene

In order to generate a DNA cassette encoding aa 44-64 of hLT-B, the *eltB* gene was cloned from human enterotoxigenic *E. coli* strain, E248750-1.

Total plasmid DNA from E248750-1 was digested with *Hin*dIII and *Eco*RI and cloned between the *Hin*dIII and *Eco*RI sites of pGEM-7zf (Fig. 6.8). After transformation of the ligation reaction into *E. coli* K-12 strain DH5α, transformants were screened on X-gal plates and tested for reactivity with a DNA probe, Dig-labelled oligo #302, based on a known internal sequence within the *eltB* gene. Ten white, DNA probe positive colonies were selected and the size of inserted DNA confirmed by restriction analysis. One clone, pPM4581, with a DNA insert of the correct size was chosen for analysis by automated Dye terminator sequencing. The sequence of the cloned *eltB* gene had 99% identity with h*eltB* and 98.5% with p*eltB* in the 588bp *Eco*RI-*Hin*dIII fragment (Fig 6.9) (Leong *et al.*, 1985; Dallas and Falkow, 1988). The predicted

Fig. 6.6 Western blot analysis of mutant CstH proteins with a polyclonal antiserum to CS3.

Cell lysates of *E. coli* K-12 expressing CstH and mutant CstH proteins were electrophoresed in SDS on 15% PAGE and transferred to nitrocellulose. A polyclonal antiserum against purified CS3 was used to probe for CstH and its mutants and visualized by ECL.

Lanes:

1. Purified CS3 (+control)
2. PB176p$^-$ (-control)
3. PB176p (+control)
4. *E. coli* K-12[pPM484] (+control)
5. *E. coli* K-12[pPM4555]expressing CstH from PB176
6. *E. coli* K12 [pPM4556] expressing mutant CstH (aa 66 and 67)
7. *E. coli* K-12 [pPM4557] expressing mutant CstH (aa 100)
8. *E. coli* K-12 [pPM4559]expressing mutant CstH (aa109)

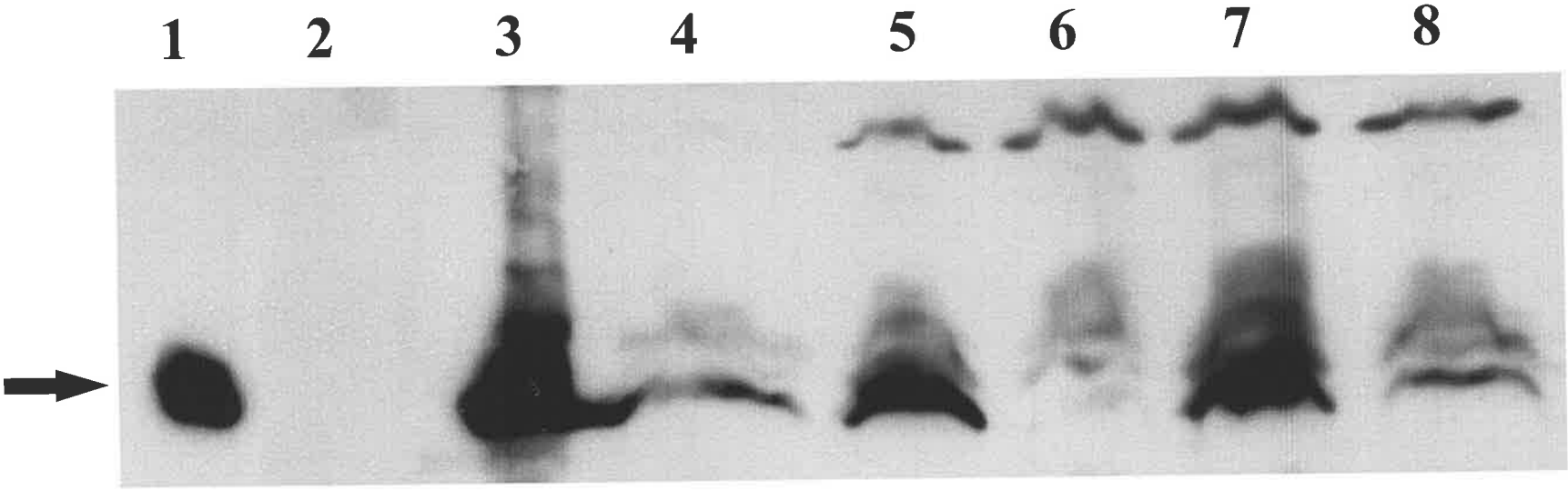

1
2
3
4
5
6
7
8

Fig. **6.7** Western blot analysis of mutant CstH proteins with Mabs 10:2 and 11:2.

Cell lysates of the strains were electrophoresed in SDS on 15% PAGE, transferred to a nitrocellulose filter and probed with Mabs 10:2 and 11:2 and visualized byECL dctcction. Wild type ETEC strain, PB176, and *E. coli* K-12 [pPM484] were used as positive controls. The arrows indicate the position of CstH.

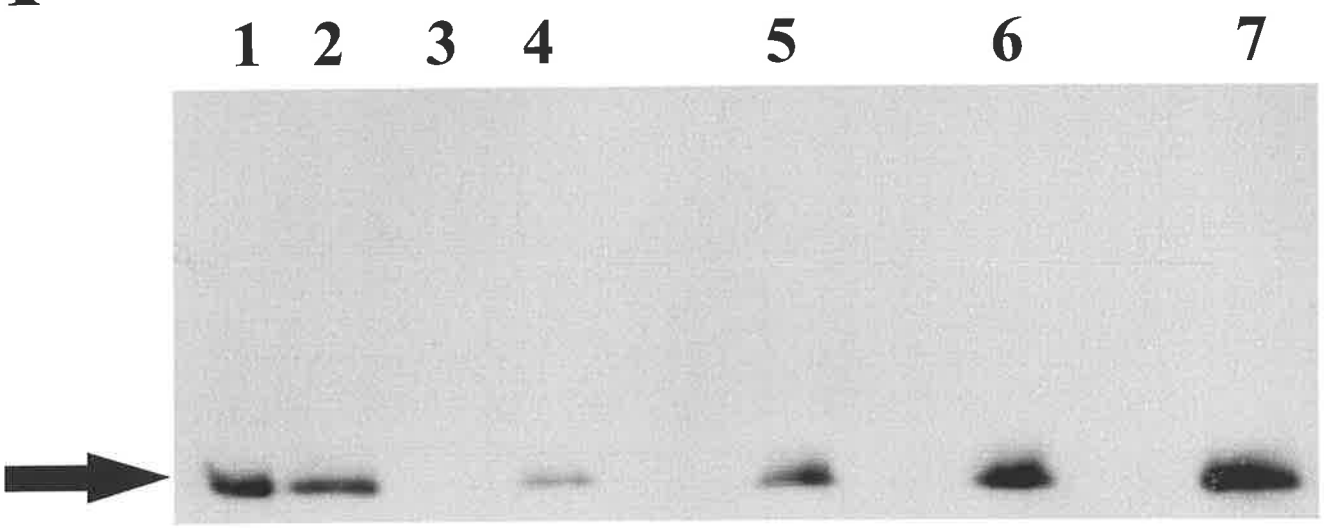
Panel A: Mab 10:2

Panel B: Mab 11:2

Lanes:

1. PB176 (+control)
2. *E. coli* K-12[pPM484] (+control)
3. PB176p$^-$ (-control)
4. *E. coli* K-12[pPM4555] expressing CstH from PB176
5. *E. coli* K-12[pPM4556] expressing mutant CstH (aa 66 and 67)
6. *E. coli* K-12[pPM4557] expressing mutant CstH (aa 100)
7. *E. coli* K-12[pPM4559] expressing mutant CstH (aa109)

A



B

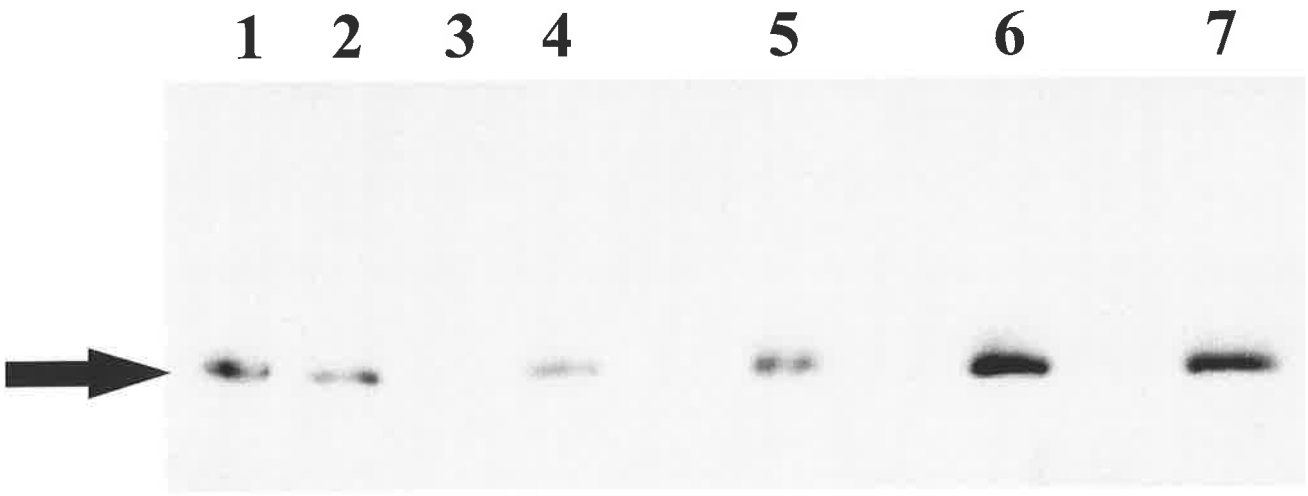


Fig. 6-8 Construction of plasmid pPM4581.

Plasmid DNA from E248750-1 was digested with *Hin*dIII and *Eco*RI and the fragments ligated between the *Hin*dIII and *Eco*RI sites of pGEM-7zf. Transformants were selected by blue/white colour selection on X-gal plates and Ap^R and confirmed by DNA sequencing. The plasmid carrying the *eltB* gene was designated pPM4581.

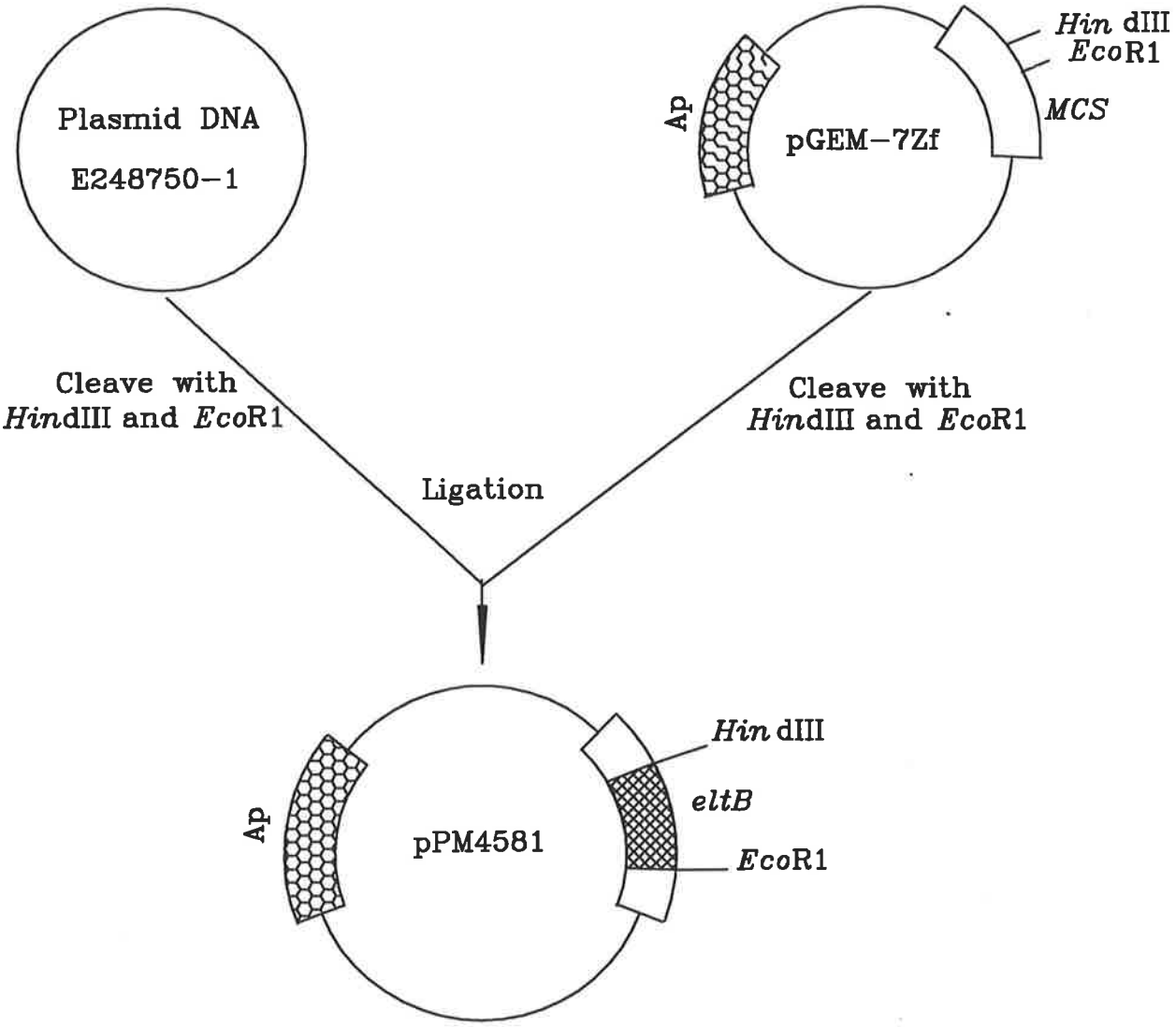

Plasmid DNA
E248750−1
Cleave with
*Hin*dIII and *Eco*R1
pGEM−7Zf
Ap
Hin dIII
*Eco*R1
MCS
Cleave with
*Hin*dIII and *Eco*R1
Ligation
pPM4581
Ap
Hin dIII
eltB
*Eco*R1

Fig. 6.9 DNA and amino acid sequence homology of the cloned *eltB* gene to known sequences

The DNA (Panel A) and amino acid (Panel B) sequence of the cloned *eltB* gene was compared with those of h*eltB* and p*eltB* using the CLUSTAL V program. Numbers indicate the position of the nucleotide and amino acids. The cloned *eltB* showed 99% and 98.5% homology with the h*eltB* and p*eltB* at the nucleotide level, respectively. The stars indicate identity, dots similarity and differences have been shown by space.

Panel A

Nucleotide sequence alignment of the cloned *eltB* gene to known sequences

```
                1                                                   50
celtB           GAATTCGGGATGAATTATGAATAAAGTAAAATATTATGTTTTATTTACGG
heltB           GAATTCGGGATGAATTATGAATAAAGTAAAATTTTATGTTTTATTTACGG
peltB           GAATTCGGGATGAATTATGAATAAAGTAAAATGTTATGTTTTATTTACGG
                ********************************* *****************
                                                                   100
celtB           CGTTACTATCCTCTCTATGTGCACACGGAGCTCCCCAGTCTATTACAGAA
heltB           CGTTACTATCCTCTCTATGTGCACACGGAGCTCCTCAGTCTATTACAGAA
peltB           CGTTACTATCCTCTCTATATGCACACGGAGCTCCCCAGACTATTACAGAA
                ****************** *************** *** ***********
                                                                   150
celtB           CTATGTTCGGAATATCGCAACACACAAATATATACGATAAATGACAAGAT
heltB           CTATGTTCGGAATATCACAACACACAAATATATACGATAAATGACAAGAT
peltB           CTATGTTCGGAATATCGCAACACACAAATATATACGATAAATGACAAGAT
                ***************** **********************************
                                                                   200
deltB           ACTATCATATACGGAATCGATGGCAGGCAAAAGAGAAATGGTTATCATTA
heltB           ACTATCATATACGGAATCGATGGCAGGCAAAAGAGAAATGGTTATCATTA
peltB           ACTATCATATACGGAATCGATGGCAGGCAAAAGAGAAATGGTTATCATTA
                **************************************************
                                                                   250
celtB           CATTTAAGAGCGGCGCAACATTTCAGGTCGAAGTCCCGGGCAGTCAACAT
heltB           CATTTAAGAGCGGCGCAACATTTCAGGTCGAAGTCCCGGGCAGTCAACAT
peltB           CATTTAAGAGCGGCGAAACATTTCAGGTCGAAGTCCCGGGCAGTCAACAT
                *************** **********************************
                                                                   300
celtB           ATAGACTCCCAAAAAAAAGCCATTGAAAGGATGAAGGACACATTAAGAAT
heltB           ATAGACTCCCAAAAAAAAGCCATTGAAAGGATGAAGGACACATTAAGAAT
peltB           ATAGACTCCCAGAAAAAAGCCATTGAAAGGATGAAGGACACATTAAGAAT
                *********** ***************************************
                                                                   350
celtB           CACATATCTGACCGAGACCAAAATTGATAAATTATGTGTATGGAATAATA
heltB           CACATATCTGACCGAGACCAAAATTGATAAATTATGTGTATGGAATAATA
peltB           CACATATCTGACCGAGACCAAAATTGATAAATTATGTGTATGGAATAATA
                **************************************************
                                                                   400
celtB           AAACCCCCAATTCAATTGCGGCAATCAGTATGGAAAACTAGTTTGCTTTA
heltB           AAACCCCCAATTCAATTGCGGCAATCAGTATGGAAAACTAGTTTGCTTTA
peltB           AAACCCCCAATTCAATTGCGGCAATCAGTATGAAAAACTAGTTTGCTTTA
                ********************************* *****************
                                                                   450
celtB           AAAGCATGTCTAATGCTAGGAGCCTATATAACAACTACTGTACTTATACT
heltB           AAAGCATGTCTAATGCTAGGAACCTATATAACAACTACTGTACTTATACT
peltB           AAAGCATGTCTAATGCTAGGAACCTATATAACAACTACTGTACTTATACT
                ********************* ****************************
                                                                   500
celtB           AATGAGCCTTATGCTGCATTTGAAAAGGCGGTAGAGGATGCAATACCGAT
heltB           AATGAGCCTTATGCTGCATTTGAAAAGGCGGTAGAGGATGCAATACCGAT
peltB           AATGAGCCTTATGCTGCATTTGAAAAGGCGGTAGAGGAGGCAATACCGAT
                ************************************ ***********
                                                                   550
celtB           CCTTAAACTGTAACACTATAACAGCTTCCACTACAGGGAGCTGTTATAGC
heltB           CCTTAAACTGTAACACTATAACAGCTTCCACTACAGGGAGCTGTTATAGC
peltB           CCTTAAACTGTAACACTATAACAGCTTCCACTACAGGGAGCTGTTATAGC
                **************************************************
                                                              588
celtB           ACACAGAAAAAACTAAGCTAGGCTGGGGGG-CAAGCTT
heltB           AAACAGAAAAAACTAAGCTAGGCTGGGGGGGCAAGCTT
peltB           ACACAGAAAAAACTAAGCTAGGCTGGAGGGGCAAGCTT
                * ************************ *** *******
```

Panel B

Amino acid sequence alignment of the cloned LT-B to known sequences

```
                -21          -10          1                                  29
CLT-B            MNKVKYYVLFTALLSSLCAHGAPQSITELCSEYRNTQIYTINDKILSYTE
HLT-B            MNKVKFYVLFTALLSSLCAHGAPQSITELCSEYHNTQIYTINDKILSYTE
PLT-B            MNKVKCYVLFTALLSSLYAHGAPQTITELCSEYRNTQIYTINDKILSYTE
                 ***** *********** ******.********.****************
                30                                                  79
CLT-B            SMAGKREMVIITFKSGATFQVEVPGSQHIDSQKKAIERMKDTLRITYLTE
HLT-B            SMAGKREMVIITFKSGATFQVEVPGSQHIDSQKKAIERMKDTLRITYLTE
PLT-B            SMAGKREMVIITFKSGETFQVEVPGSQHIDSQKKAIERMKDTLRITYLTE
                 **************** **********************************
                80                          103
CLT-B            TKIDKLCVWNNKTPNSIAAISMEN
HLT-B            TKIDKLCVWNNKTPNSIAAISMEN
PLT-B            TKIDKLCVWNNKTPNSIAAISMKN
                 ********************** *
```

amino acid sequence of LT-B from the cloned *eltB* gene showed 98.4% identity with hLT-B and 96.1% with pLT-B (Table 6.1). Plasmid pPM4581 was used for the generation of the *eltB* epitope cassette.

6.2.4 Construction of an *eltB* epitope cassette and cloning into *cstH*

In order to construct the *eltB* epitope cassette, a DNA fragment encoding amino acids 44-64 (LT-B_{44-64}) was amplified by PCR. Oligos #915 and #916, containing *Sty*I sites, and corresponding to nt 197-220 and nt 259-285 of *eltB,* were designed in such a way that they would allow in-frame insertion of the epitope-encoding fragment into *cstH.* PCR was carried out using these oligos as primers to amplify a fragment of 89 bp. The PCR product was purified, digested with *Sty*I, and the fragment was cloned into either *Sty*I or *Xba*I sites of the mutant *cstH* genes, between nt 4413-4414 and 4515-4516, of plasmids pPM4556 and pPM4557, respectively (Fig. 6.10). Insertion between nt 4542 and 4543 of *cstH'* in pPM4559 was not successful. Transformants were screened using an internal DNA probe (oligo #917). The in-frame insertion and the orientation of the foreign epitope in *cstH* were confirmed by sequencing of the fusions. The correct constructs were designated as pPM4585 and pPM4589, respectively, and used for further investigation.

Computer analysis of the secondary structure and solvent accessibility of the hybrid proteins using the PHD program suggested that the foreign epitope will be present as a loop and exposed to the environment (Fig. 6.11). The data predict that the insert would be expressed on the surface of the protein and eventually on the surface of the hybrid pili and the bacteria producing them.

Table 6.1 Comparison of the cloned *eltB* in pPM4581 with those ETEC strains

Strain	seq.differences	Corresponding aa differences	Overall % homology Nucleotide seq.	amino acid seq.
h*eltB*	$A_{32}\rightarrow T$	$Tyr_{10}\rightarrow Phe$	99.2	98.4
	$C_{84}\rightarrow T$	$Arg_{38}\rightarrow His$		
	$G_{116}\rightarrow A$			
p*eltB*	$A_{32}\rightarrow G$	$Tyr_{10}\rightarrow Cys$	98.5	96.1
	$G_{68}\rightarrow A$	$Cys_{22}\rightarrow Tyr$		
	$T_{88}\rightarrow A$	$Ser_{29}\rightarrow Thr$		
	$C_{215}\rightarrow A$	$Ala_{71}\rightarrow Glu$		
	$A_{261}\rightarrow G$	$Glu_{127}\rightarrow Lys$		
	$G_{382}\rightarrow A$			

Fig. 6-10 Insertion of *eltB*$_{44\text{-}64}$ into *cstH*.

A 89 bp fragment encoding amino acid residues 44-64 of LT-B was amplified by PCR using oligos #915 and #916. The DNA fragment was digested with *Sty*I and ligated into the *Sty*I or *Xba*I sites of the mutated *cstH* genes. Transformants were screened by DNA probing using Dig-labelled oligo #312 and confirmed by sequencing. The plasmids designated pPM4585 and pPM4589 were generated from plasmids pPM4556 and pPM4557, respectively.

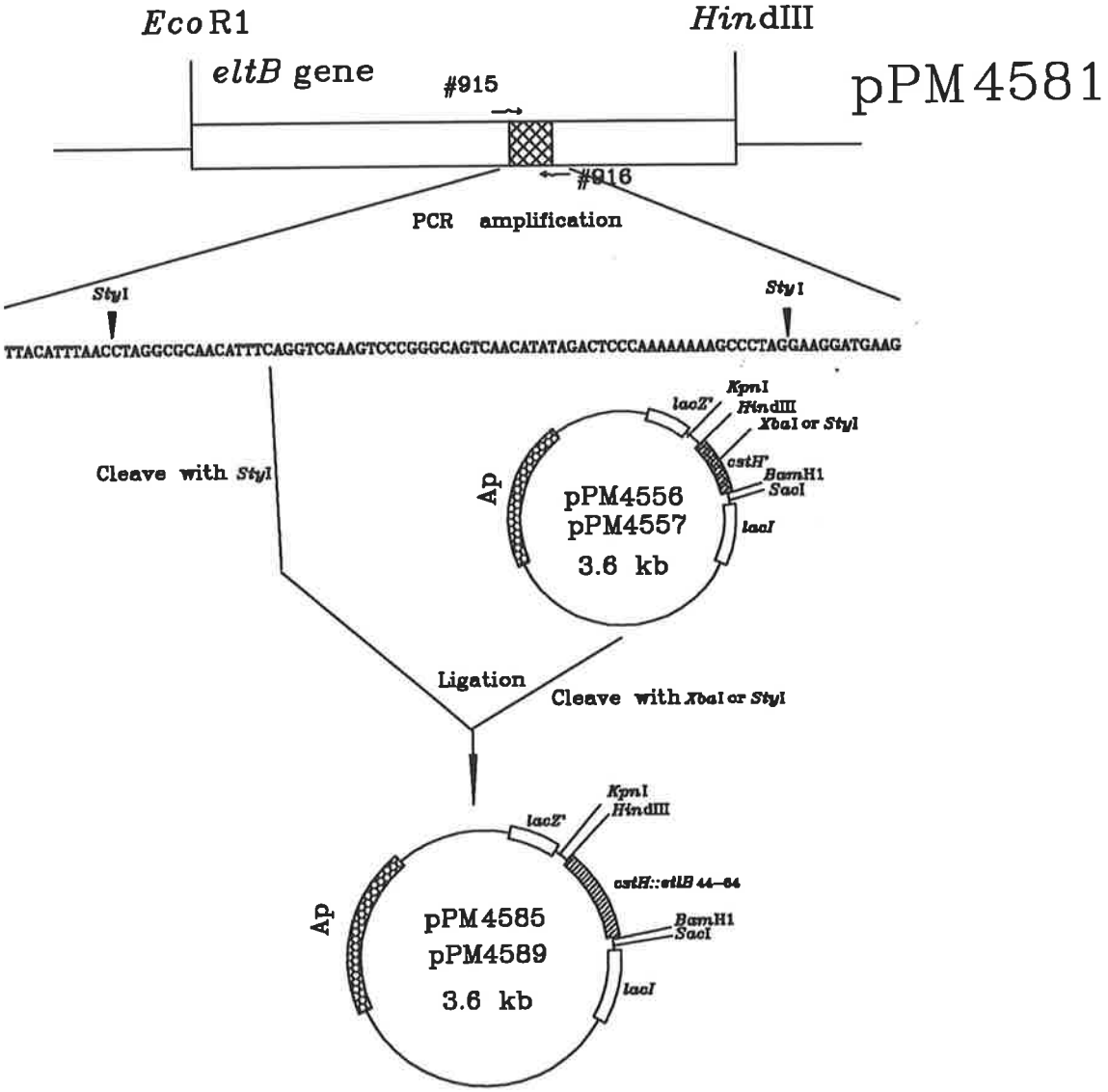
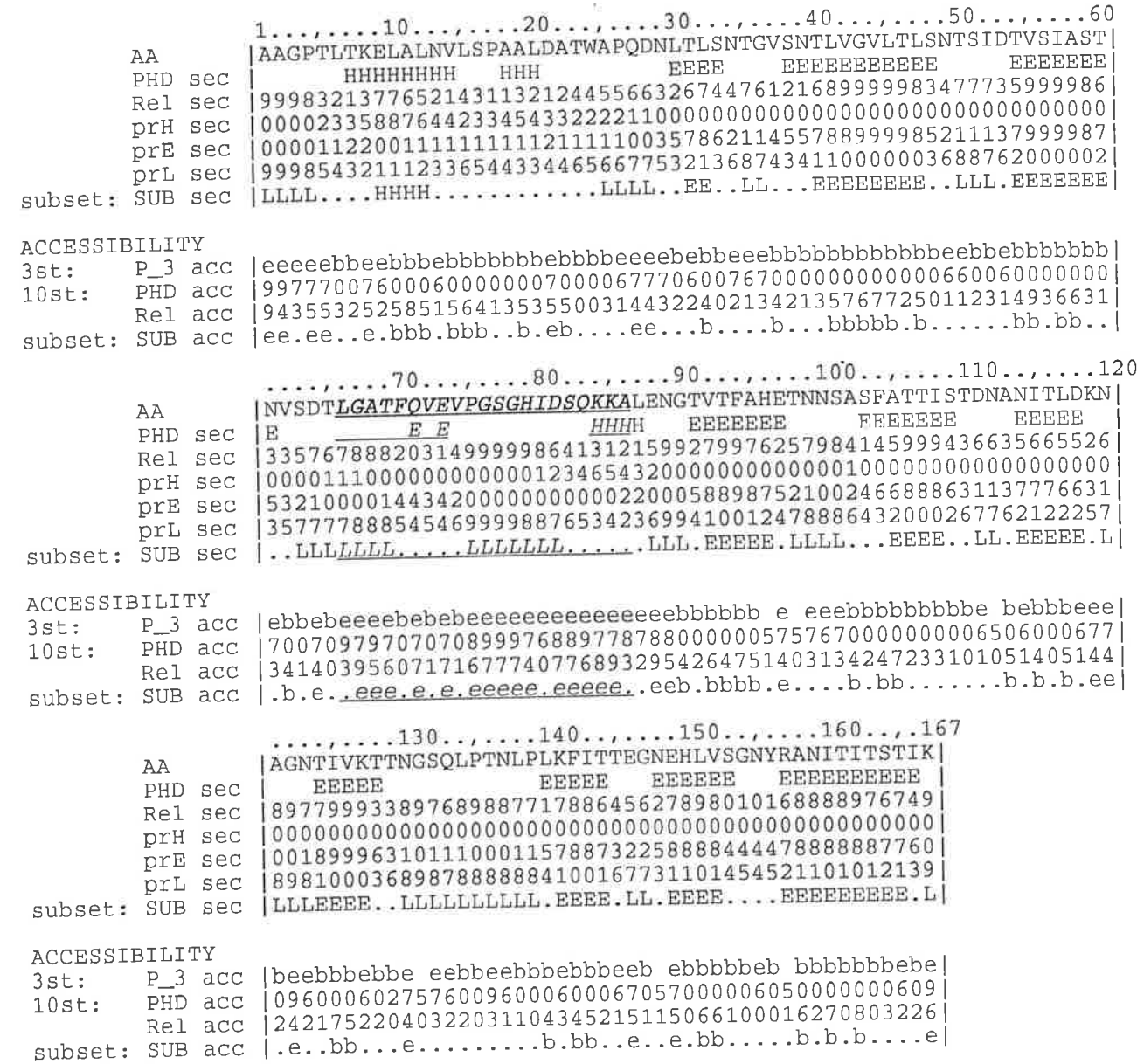

EcoR1
HindIII
eltB gene
#915
pPM4581
#916
PCR amplification
StyI
StyI
TTACATTTAACCTAGGCGCAACATTTCAGGTCGAAGTCCCGGGCAGTCAACATATAGACTCCCAAAAAAAAGCCCTAGGAAGGATGAAG
Cleave with StyI
KpnI
lacZ'
HindIII
XbaI or StyI
cstH'
BamH1
SacI
lacI
Ap
pPM4556
pPM4557
3.6 kb
Ligation
Cleave with XbaI or StyI
KpnI
HindIII
lacZ'
cstH::eltB 44–64
BamH1
SacI
lacI
Ap
pPM4585
pPM4589
3.6 kb

Fig. 6.11 Secondary structure analysis of the amino acid sequence of the CstH::LT-B_{44-64} hybrid proteins

The secondary structure and solvent accessibility of the CstH::LT-B_{44-64} hybrid proteins was determined using the PHD program (Rost and Sander, 1993; 1994). Panel A indicates the hybrid protein with insert between amino acids 65-66 (pPM4585), and panel B with the insert between amino acids 99-100 (pPM4589). The underlined, italic sequences correspond to the inserts. The abbreviations are the same as in Fig. 6.4.

Panel A


1...,....10...,....20...,....30...,....40...,....50...,....60
AA |AAGPTLTKELALNVLSPAALDATWAPQDNLTLSNTGVSNTLVGVLTLSNTSIDTVSIAST|
PHD sec | HHHHHHHH HHH EEEE EEEEEEEEEEE EEEEEEE|
Rel sec |999832137765214311321244556632674476121689999983477735999986|
prH sec |000023358876442334543322221100000000000000000000000000000000|
prE sec |000011220011111111112111110035786211455788999985211137999987|
prL sec |999854321112336544334465667753213687434110000003688762000002|
subset: SUB sec |LLLL....HHHH.....LLLL..EE..LL...EEEEEEEE..LLL.EEEEEEE|
ACCESSIBILITY
3st: P_3 acc |eeeeebbeebbbebbbbbbbebbbbeeeebebbeeebbbbbbbbbbbbeebbebbbbbbb|
10st: PHD acc |997770076000600000007000067770600767000000000006600600000000|
Rel acc |943553252585156413535500314432240213421357677250112314936631|
subset: SUB acc |ee.ee..e.bbb.bbb..b.eb....ee...b....b...bbbbb.b......bb.bb..|
....,....70...,....80...,....90...,....100..,....110..,....120
AA |NVSDTLGATFQVEVPGSGHIDSQKKALENGTVTFAHETNNSASFATTISTDNANITLDKN|
PHD sec |E E E HHHH EEEEEEE EEEEEEE EEEEE |
Rel sec |335767888203149999986413121599279976257984145999436635665526|
prH sec |000011100000000000012346543200000000000001000000000000000000|
prE sec |532100001443420000000000220005889875210024668886311377766 31|
prL sec |357777888545469999887653423699410012478886432000267762122257|
subset: SUB sec |..LLLLLLLL.....LLLLLLL.....LLL.EEEEE.LLLL...EEEE..LL.EEEEE.L|
ACCESSIBILITY
3st: P_3 acc |ebbebeeeebebebeeeeeeeeeeeeeeebbbbbb e eeebbbbbbbbbe bebbbeee|
10st: PHD acc |700709797070708999768897787880000005757670000000006506000677|
Rel acc |341403956071716777407768932954264751403134247233101051405144|
subset: SUB acc |.b.e..eee.e.e.eeeee.eeeee..eeb.bbbb.e....b.bb.....b.b.b.ee|
....,....130..,....140..,....150..,....160..,.167
AA |AGNTIVKTTNGSQLPTNLPLKFITTEGNEHLVSGNYRANITITSTIK|
PHD sec | EEEEE EEEEE EEEEEE EEEEEEEEEE |
Rel sec |897799933897689887717886456278980101688889767 49|
prH sec |000000000000000000000000000000000000000000000000|
prE sec |001899963101110001157887322588884444788888877 60|
prL sec |898100036898788888841001677311014545211010121 39|
subset: SUB sec |LLLEEEE..LLLLLLLLLL.EEEE.LL.EEEE....EEEEEEEEE.L|
ACCESSIBILITY
3st: P_3 acc |beebbbebbe eebbeebbbebbbeeb ebbbbbeb bbbbbbbebe|
10st: PHD acc |096000602757600960006000670570000060500000006 09|
Rel acc |242175220403220311043452151150661000162708032 26|
subset: SUB acc |.e..bb...e.....b.bb..e..e.bb.....b.b.b....e|

Panel B

```
                 ....,....10...,....20...,....30...,....40...,....50...,....60
          AA     |AAGPTLTKELALNVLSPAALDATWAPQDNLTLSNTGVSNTLVGVLTLSNTSIDTVSIAST|
          PHD sec|      HHHHHHHH   HHH          EEEE    EEEEEEEEEEE     EEEEEEE|
           Rel sec|999832137765214311321244556632674476121689999983477736999975|
          prH sec|000023358876442334543322221100000000000000000000000000000000|
          prE sec|000011220011111111112111110035786211455788999985211137999886|
          prL sec|999854321112336544334465667753213687434110000003688762000012|
subset:   SUB sec|LLLL....HHHH.............LLLL..EE..LL...EEEEEEEE..LLL.EEEEEEE|

ACCESSIBILITY
3st:      P_3 acc|eeeeebbeebbbebbbbbbbebbbbeeeebebbeeebbbbbbbbbbbbeebbebbbbbbb|
10st:     PHD acc|997770076000600000007000067770600767000000000006600600000000|
          Rel acc|943553252585156413535500314432240213421357687250112314936630|
subset:   SUB acc|ee.ee..e.bbb.bbb..b.eb....ee...b....b...bbbbb.b......bb.bb..|
                  ....,....70...,....80...,....90...,....100..,....110..,....120
          AA     |NVSDTSKNGTVTFAHETNNSASFATTISTDNANITLDKNLGATFQVEVPGSGHIDSQKKA|
          PHD sec|          EEEEEEE      EEEEEE    EEEE      E  E          HHHH|
          Rel sec|137689998799986257975144999446634664378999721224999998641321|
          prH sec|000000000000000000000000000000000000001100000100000001225554|
          prE sec|431100001799987521012466888621136776310000154352000000000001|
          prL sec|567789888100012478886422000367752112678899744546999998764333|
subset:   SUB sec|..LLLLLLLEEEEEE.LLLLL...EEE..LL..EE..LLLLLL.....LLLLLLL...../

ACCESSIBILITY
3st:      P_3 acc|ebeebbeebbbbbb e eeebbbbbbbbbe bebbbbeeeeeebebebeeeeeeeeeeee|
10st:     PHD acc|707700780000005757670000000006506000077697707070899976888779|
          Rel acc|332401661052751403134247233101051516044094606261677740776794|
subset:   SUB acc|...e..ee..b.bb.e....b.bb.......b.b.b.ee.eee.e.e.eeeee.eeeeee|
                  ....,....130..,....140..,....150..,....160..,.167
          AA     |LGNTIVKTTNGSQLPTNLPLKFITTEGNEHLVSGNYRANITITSTIK|
          PHD sec|   EEEEE           EEEEE   EEEEEE   EEEEEEEEEE |
          Rel sec|589289915798689887717886456278980101688889767 49|
          prH sec|100000000000000000000000000000000000000000000000|
          prE sec|110589852101110001157887322588884444788888877 60|
          prL sec|688300047898788888841001677311014545211010121 39|
subset:   SUB sec|LLL.EEE.LLLLLLLLLLL.EEEE.LL.EEEE....EEEEEEEEE.L|

ACCESSIBILITY
3st:      P_3 acc|bbebbbebbe eebbeebbbebbbeeb ebbbbbeb bbbbbbbebe|
10st:     PHD acc|006000602757600960006000670570000006050000000609|
          Rel acc|011376220403220311043452151150661000162709032 26|
subset:   SUB acc|....bb...e.........b.bb..e..e.bb.....b.b.b....e|
```

6.2.5 Characterization of CstH::LT-B_{44-64} hybrid proteins

6.2.5.1 Expression of hybrid proteins in *E. coli* K-12 strain DH5α

The expression of the hybrid proteins was analyzed using *E. coli* K-12 strain DH5α harbouring plasmids pPM4585 and pPM4589. In no case could the hybrid proteins be detected by Western blot analysis using antiserum against native CS3 (Fig. 6.12 tracks 1-6). There are several possible reasons for the failure to detect the expression of these proteins and attempts were made to overcome them by the use of different expression systems. These reasons are:

1 - The hybrid proteins are unstable and are degraded within the cell.

2 - The gene is not transcribed and translated

3 - The hybrid genes are transcribed but the mRNA is unstable.

4 - The insertion of the foreign epitope has changed the conformation of the protein so that it no longer reacts with the antibody.

6.2.5.2 Expression of hybrid proteins in a *deg*^{P} strain

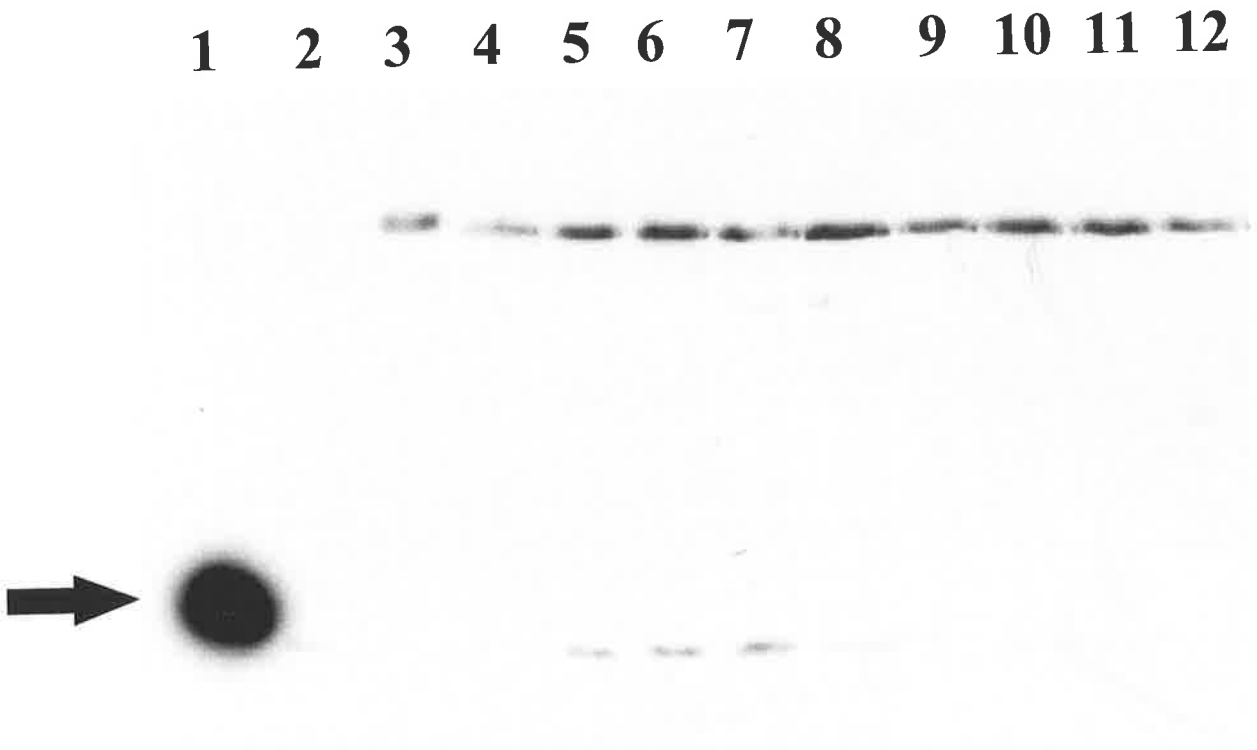
In order to protect the fusion proteins from degradation in the periplasm of the cell, the plasmids encoding the CstH::LT-B_{44-64} hybrid proteins were transformed into an *E. coli* K-12 strain (Ecc219) deficient in periplasmic degradation of proteins (Table 2.2). Since it is known that Deg^P is involved in pilin degradation (Holtgren *et al.*, 1993), this mutation might help to protect the hybrid pilin from degradation. The expression of the hybrid proteins was analyzed by Western blot using antibody against native CS3. None of the hybrid proteins could be detected (Fig. 6.12 tracks 11 and 12). Although these results suggest that the failure of the antiserum to recognize the hybrid proteins was not due to the degradation of the hybrid proteins by Deg^P, there is still the possibility that degradation may be involved.

Fig. 6.12 Western blot analysis of hybrid CstH::LT-B_{44-64} proteins.

The hybrid CstH::LT-B_{44-64} proteins were analysed by Western blot and visualized by ECL. The hybrid proteins were expressed in several different systems. Whole cell lysates were electrophoresed in SDS on 15% polyacrylamide gels, transferred to a nitrocellulose filter and probed with an antiserum against native CS3 and the blots were visualized by ECL. The arrow corresponds to mature CstH. Ecc 219 is the *degP* strain

Lanes:

1. PB176 (+control)
2. PB176p$^-$ (-control)
3. *E. coli* K-12[pBluescript] vector plasmid for *cstH::eltB*$_{44-64}$ (-control)
4. *E. coli* K-12[pPM4581] encoding LT-B
5. *E. coli* K-12[pPM4585] encoding CstH::LT-B_{44-64} (between aa 65-66)
6. *E. coli* K-12[pPM4589] encoding CstH::LT-B_{44-64} (between aa 99-100)
7. *E. coli* K12[pPM4585] encoding CstH::LT-B_{44-64} (between aa 65-66) and CS3 assembly genes
8. *E. coli* K-12[pPM4589] encoding CstH::LT-B_{44-64} (between aa 99-100) and CS3 assembly genes
9. PB176p$^-$[pPM4585] encoding CstH::LT-B_{44-64} (between aa 65-66) and CS3 assembly genes
10. PB176p$^-$[pPM4589] encoding CstH::LT-B_{44-64} (between aa 99-100) and CS3 assembly genes
11. *E. coli* K-12 Ecc219[pPM4585] encoding CstH::LT-B_{44-64} (between aa 65-66)
12. *E. coli* K-12 Ecc219[pPM4589] encoding CstH::LT-B_{44-64} (between aa 99-100)


1 2 3 4 5 6 7 8 9 10 11 12

6.2.5.3 Expression of hybrid proteins in complementation with the assembly genes

A wild type ETEC strain deficient in the expression of CS3, and *E. coli* K-12 strain DH5α were used to express the CstH-hybrid proteins in the presence of the CS3 assembly cassette. It was thought possible that the assembly proteins, in particular the chaperone CstA, may protect the fusion proteins from degradation within the cell (Holtgren *et al.*, 1993). Plasmids pPM4585 and pPM4589, encoding CstH::LT-$B_{44\ 64}$ hybrid proteins and pPM4551, encoding the CS3 assembly proteins were electroporated into the strains. The assembly and fusion proteins are encoded by compatible plasmids. Transformants were screened for ampicillin and chloramphenicol resistance, and the presence of both plasmids confirmed by restriction enzyme analysis. Cells harbouring both plasmids were analyzed for the expression of the hybrid proteins by Western blot analysis using an antiserum against native CS3. In no case could the hybrid proteins be detected (Fig. 6.12 tracks 7-10). Thus, it was decided to use the T7 promoter/RNA polymerase system (Tabor and Richardson, 1985) for expression of the hybrid proteins.

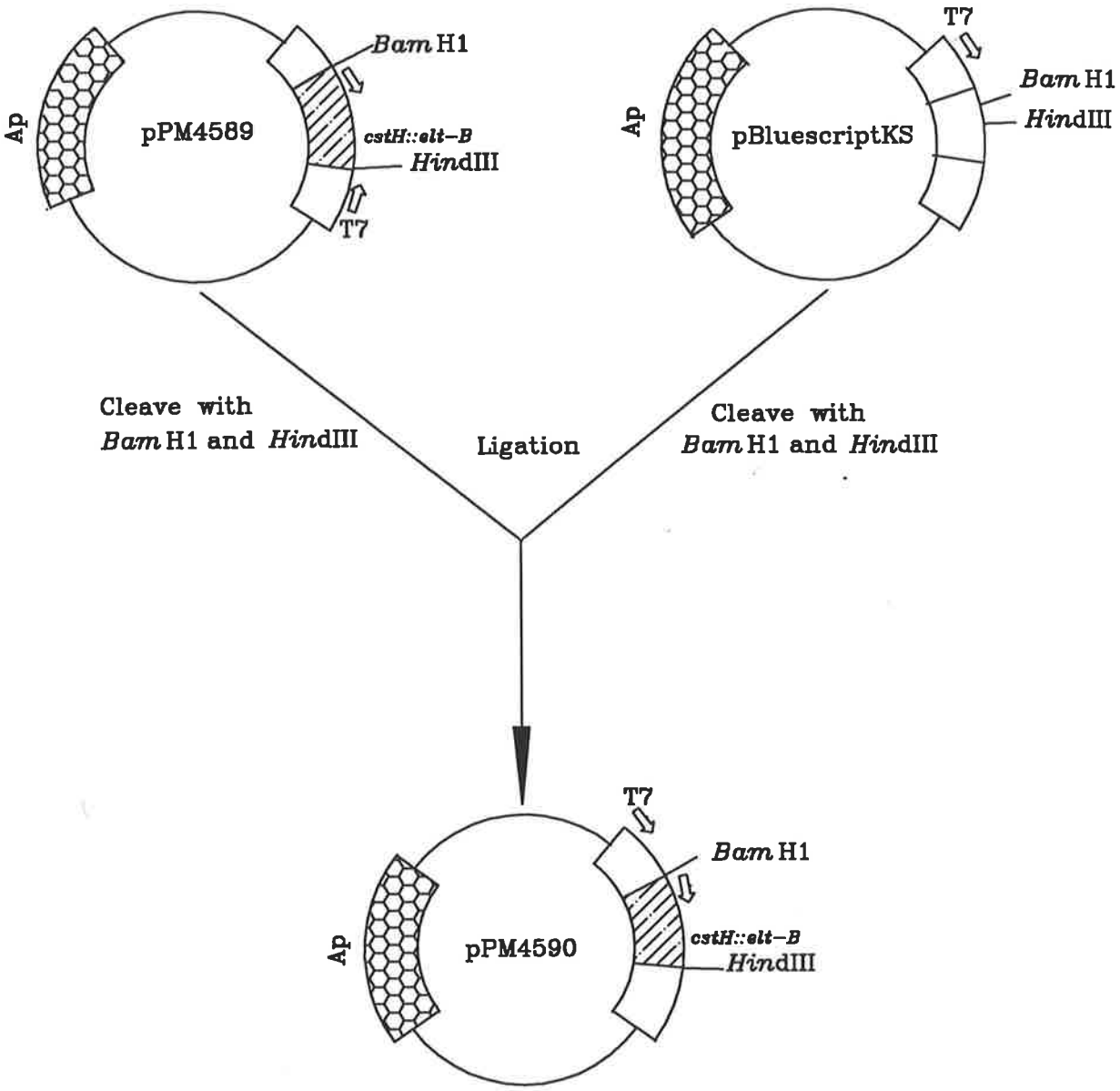
6.2.5.4 Overexpression of the hybrid proteins in the T7 promoter/RNA polymerase system

The DNA fragment encoding the hybrid protein with LT-B_{44-64} inserted between aa 99 and 100 of the mature CstH protein was not in the correct orientation for expression from the T7 promoter, consequently it was subcloned into plasmid pBluescript KS in the correct orientation to give pPM4590 (Fig. 6.13).

Plasmids pPM4585 and pPM4590 containing fragments encoding hybrid proteins, and plasmid pGP1-2 encoding the T7 RNA polymerase were transformed into *E. coli* K-12 strains MZ1 (Zuber *et al.*, 1987) and the deg^P strain Ecc219 (Table 2.2).

Fig. 6-13 Cloning of *cstH::eltB*$_{44\text{-}64}$ under the control of the T7 promoter.

Plasmid pPM4589 (based upon pBluescript SK) was digested with *Bam*HI and *Hin*dIII and the DNA fragment was ligated between *Bam*HI and *Hin*dIII sites of pBluescript KS to place *cstH::eltB*$_{44\text{-}64}$ under the control of the T7 promoter. This plasmid was designated pPM4590. The arrows indicate the orientation of the T7 promoter and *cstH* gene.


Ap
pPM4589
Bam H1
cstH::elt-B
HindIII
T7
Ap
pBluescriptKS
T7
Bam H1
HindIII
Cleave with
Bam H1 and HindIII
Ligation
Cleave with
Bam H1 and HindIII
T7
Bam H1
Ap
pPM4590
cstH::elt-B
HindIII

Transformants were selected for resistance to Ap and Km, and the presence of the two plasmids was confirmed by restriction enzyme analyses.

Expression of hybrid proteins was studied by Western blot analysis. Both hybrid proteins (LT-B_{44-64} between residues #65-#66 and #99-#100 of mature CstH) reacted with polyclonal and monoclonal antibodies raised against CS3 (Fig. 6.14). This result shows that a foreign epitope can be inserted into CstH in these regions without dramatic changes in its overall structure. However polyclonal antibodies against native CT (provided by S. Attridge) and Mab to LT-B (Mab39, provided by A.M. Svennerholm) could not recognize the hybrid proteins in Western blot analysis (Fig. 6.15).

6.2.6.1 Cloning of the hybrid *cstH* gene into the *cst* operon

It has been shown (see above) that the expression of the hybrid proteins in different systems is poor and only the use of a strong promoter provided sufficient levels of protein to permit them to be detected. It was thought that the normal biosynthetic operon structure of CS3 may help overcome the problem related to the expression of the hybrid proteins. Therefore the DNA fragments encoding the hybrid proteins were used to replace the wild type *cstH* in the *cst* operon.

Plasmids pPM4585, pPM4589 (encoding CstH::LT-B_{44-64} hybrid proteins) and pPM484 were digested with *Bsu*36I and *Bam*HI and the 4.3 kb DNA fragment from pPM484 containing the CS3 assembly genes was cloned between the *Bsu*36I and *Bam*HI sites of plasmids pPM4585 and pPM4589, to give plasmids pPM4588 and pPM4593 respectively (Fig. 6.16). In plasmid pPM4588 the reconstituted hybrid *cst* operon is orientated so that it can be expressed from the T7 promoter when necessary.

Fig. 6.14 Immunoblot analysis of hybrid CstH proteins.

The CstH::LT-B_{44-64} hybrid proteins were expressed in the T7 promoter/RNA polymerase system and analysed by Western blot and detected by ECL. Cell lysates were run in SDS on 15% polyacrylamide gels, transferred to a nitrocellulose filter and probed with a polyclonal antiserum against CS3 (panel A) and with Mab CH1 to CS3 (panel B). The arrows on the left indicate native CstH.

Lanes:

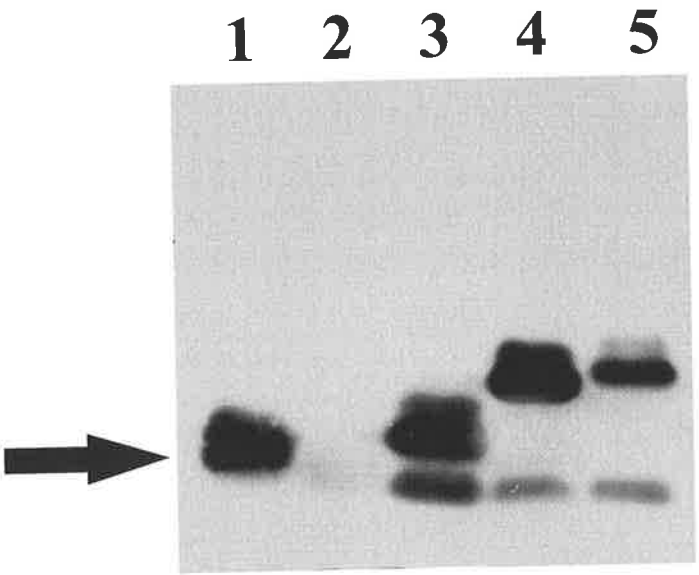
A:

1. PB176 (+control)
2. PB176p^- (-control)
3. *E. coli* K-12[pPM4556 and pGP1-2] expressing mutant CstH (between aa 65-66)
4. *E. coli* K-12[pPM4585 and pGP1-2] expressing CstH::LT-B_{44-64} (between aa 65-66)
5. *E. coli* K-12[pPM4590 and pGP1-2] expressing CstH::LT-B_{44-64} (between aa 99-100)

B:

1. PB176 (+control)
2. PB176p^- (-control)
3. *E. coli* K-12[pPM4585 and pGP1-2] expressing CstH::LT-B_{44-64} (between aa 65-66)
4. *E. coli* K-12[pPM4590 and pGP1-2] expressing CstH::LT-B_{44-64} (between aa 99-100)

A



B

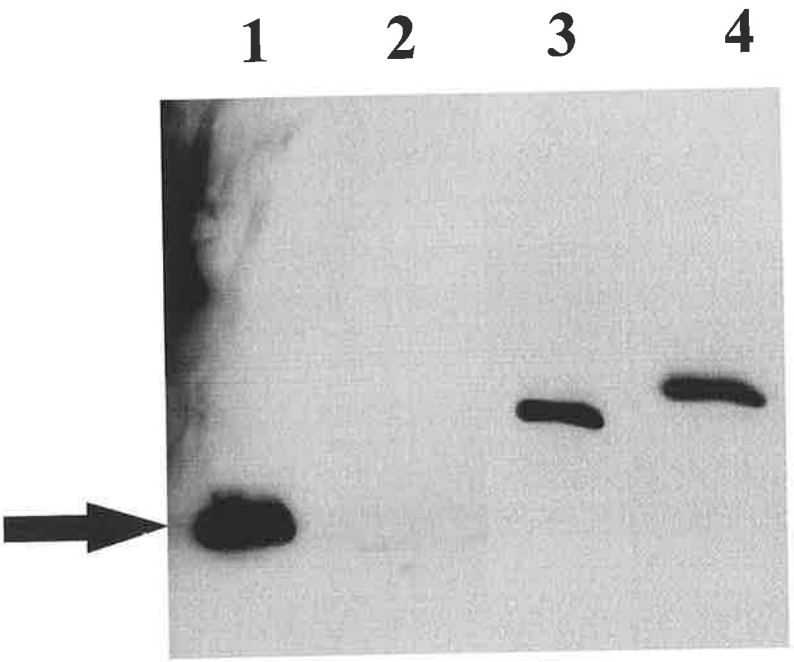
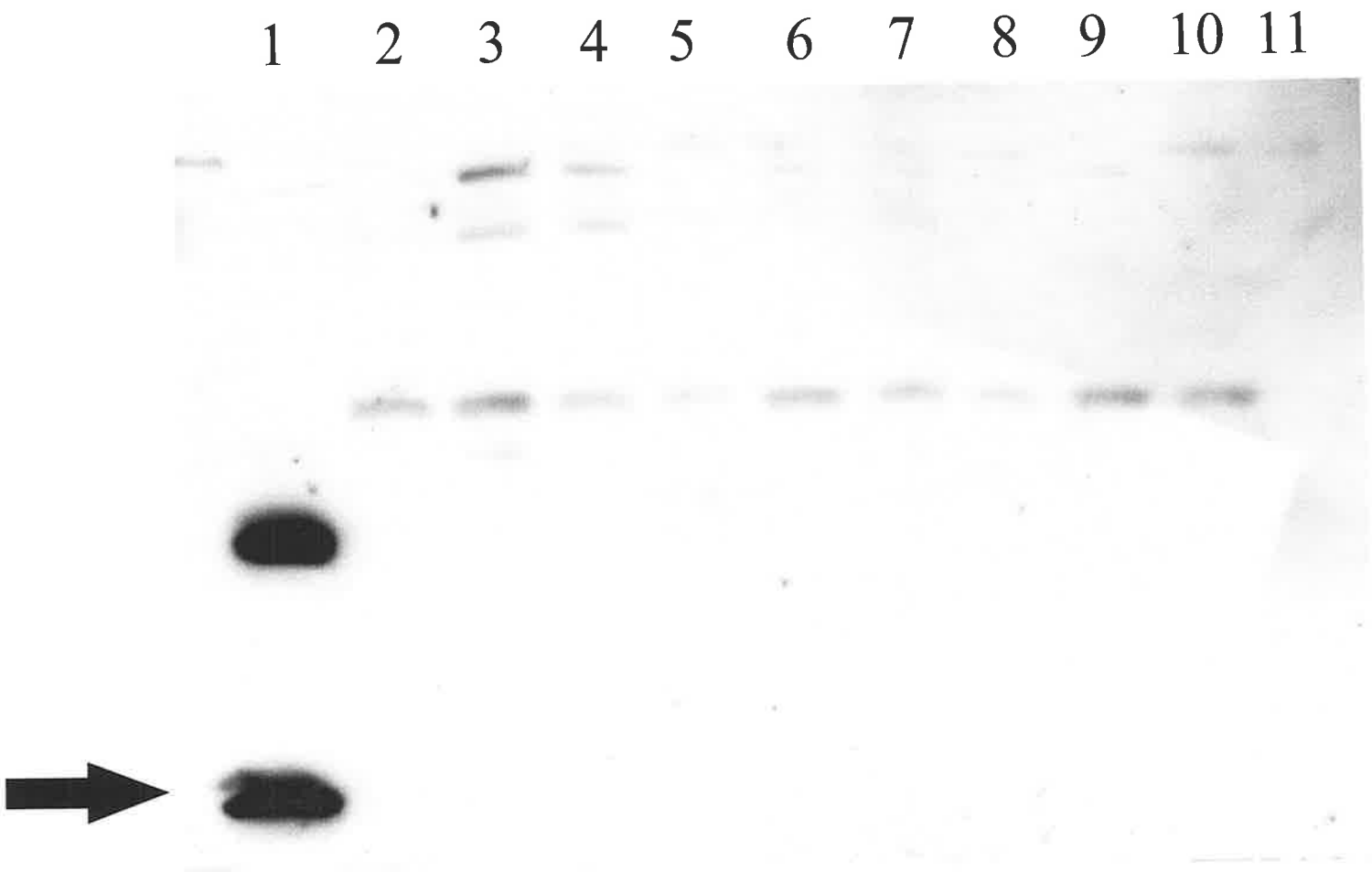


Fig. 6.15 Western blot analysis of hybrid CstH::LT-B_{44-64} proteins.

The CstH::LT-B_{44-64} hybrid proteins were expressed in different systems and analysed in Western blot and detected by ECL. Whole cell lysates were electrophoresed in SDS on 15% polyacrylamide gels, transferred to a nitrocellulose filter and probed with a polyclonal antiserum against CT. The arrow indicates CT-B.

Lanes:

1. Cholera toxin (+ control)
2. *E. coli* K-12[pBluescript] vector plasmid for *cstH::elt-*B_{44-64} (-control)
3. *E. coli* K-12[pPM4581] encoding LT-B
4. *E. coli* K-12[pPM4585] encoding CstH::LT-B_{44-64} (between aa 65-66)
5. *E. coli* K-12[pPM4589] encoding CstH::LT-B_{44-64} (between aa 99-100)
6. *E. coli* K12[pPM4585] encoding CstH::LT-B_{44-64} (between aa 65-66) and CS3 assembly genes
7. *E. coli* K-12[pPM4589] encoding CstH::LT-B_{44-64} (between aa 99-100) and CS3 assembly genes
8. PB176p$^-$[pPM4585] encoding CstH::LT-B_{44-64} (between aa 65-66) and CS3 assembly genes
9. PB176p$^-$[pPM4589] encoding CstH::LT-B_{44-64} (between aa 99-100) and CS3 assembly genes
10. *E. coli* K-12 [pPM4585 and pGP1-2] encoding CstH::LT-B_{44-64} (between aa 65-66)
11. *E. coli* K-12 [pPM4590 and pGP1-2] encoding CstH::LT-B_{44-64} (between aa 99-100)


1
2
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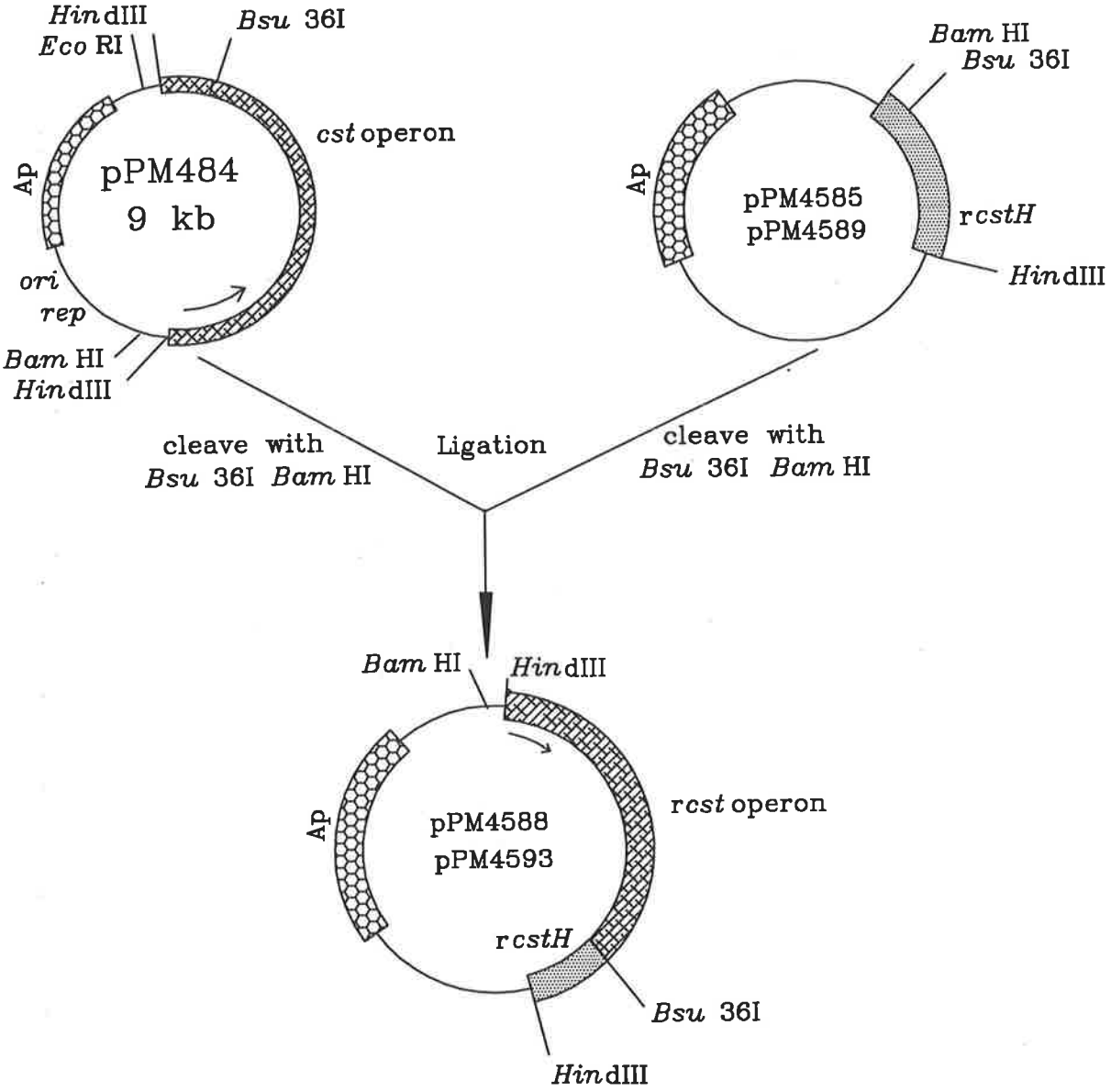
10
11

Fig 6.16 Construction of hybrid *cst* operon

Plasmids pPM484, pPM4585 and pPM4589 were digested with *Bam*HI and *Bsu*36I and the DNA fragment containing the CS3 assembly genes from pPM484 was cloned between the *Bam*HI and *Bsu*36I sites of pPM4585 and pPM4589 upstream of recombinant *cstH* gene to generate plasmids pPM4588 and pPM4593, respectively.


Hin dIII
Eco RI
Bsu 36I
cst operon
Ap
pPM484
9 kb
ori
rep
Bam HI
Hin dIII
Bam HI
Bsu 36I
Ap
pPM4585
pPM4589
rcstH
Hin dIII
cleave with
Bsu 36I Bam HI
Ligation
cleave with
Bsu 36I Bam HI
Bam HI
Hin dIII
Ap
pPM4588
pPM4593
rcst operon
rcstH
Bsu 36I
Hin dIII

6.2.6.2 Expression of the hybrid proteins from within the *cst* operon

Expression of the hybrid proteins was investigated by Western blotting using polyclonal and Mab (CH10) against CS3 and Mab against LT (Mab 39) and polyclonal antibodies against CT. Since optimal expression of the native CS3 pili is accomplished on CFA medium (Evans *et al.,* 1979) it was also used for expression of the hybrid proteins.

Bacteria harbouring plasmids pPM4588 and pPM4593, which encode the hybrid CstH::LT-$B_{44\text{-}64}$ with insertion of foreign epitope between amino acids 65-66 and 99-100 respectively, were grown overnight on CFA containing Ap. Cell lysates from 5×10^9 cells were used for Western blot analysis. Polyclonal and Mab CH10 against CS3 could detect protein with the size of the hybrid proteins (Fig 6.17). Mab to LT could not detect the hybrid proteins (the Mab is against B pentamer) (Fig. 6.18 A), but polyclonal antiserum against CT could recognize the hybrid proteins in Western blot (Fig. 6.18 B). The positions of the bands recognized by polyclonal antiserum against CT were higher than those of the bands detected by antibodies against CS3 and CstH, being approximately twice the molecular weight. These could be dimer hybrid proteins which were recognized by anti-CT antibodies, while the monomer hybrid was not. In support of this possibility, polyclonal antiserum against CS3 detected several bands some of them corresponding to the bands, recognized by anti-CT antibodies.

6.2.7 Re-cloning and expression of the mutant *cst* operon in plasmid pBC KS

While the construction of the hybrid *cst* operon was in progress, vector pBC KS which is derived from pBluescript with a Cm^R marker became available. In order to check the expression of the hybrid CstH proteins in an alternative vector, the wild type and the mutant *cst* operons were cloned into plasmid pBC KS. Purified *Hin*dIII

Fig. 6.17 Western blot analysis of hybrid CstH::LT-B_{44-64} proteins expressed by recombinant *cst* operons.

The hybrid CstH::LT-B_{44-64} proteins, expressed from a recombinant *cst* operon, were analysed in Western blot using the appropriate antibodies and detected by ECL. Whole cell lysates were electrophoresed in SDS on 15% polyacrylamide gels, transferred to a nitrocellulose filter and probed with either a polyclonal antiserum to CS3 (Panel A) or Mab CH10 to CstH (Panel B). The arrow indicates CstH.

Lanes:

A and B

1. PB176 (+control)
2. *E. coli* K-12[pBluescript]vector plasmid for *cstH::elt-*B_{44-64} (-control)
3. *E. coli* K-12[pPM484] expressing wild type CstH
4. *E. coli* K-12[pPM4588] expressing CstH::LT-B_{44-64} (between aa 65-66)
5. *E. coli* K-12[pPM4593] expressing CstH::LT-B_{44-64} (between aa 99-100)

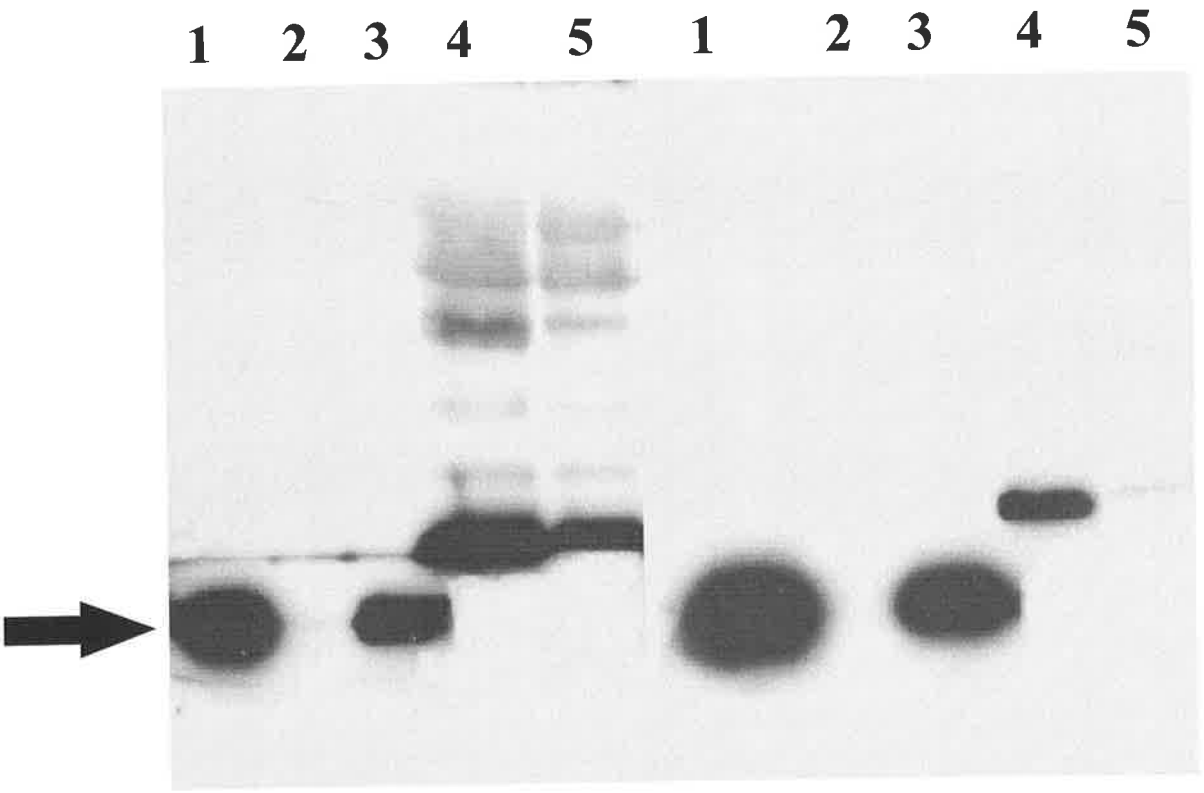

1 2 3 4 5 1 2 3 4 5
A
B

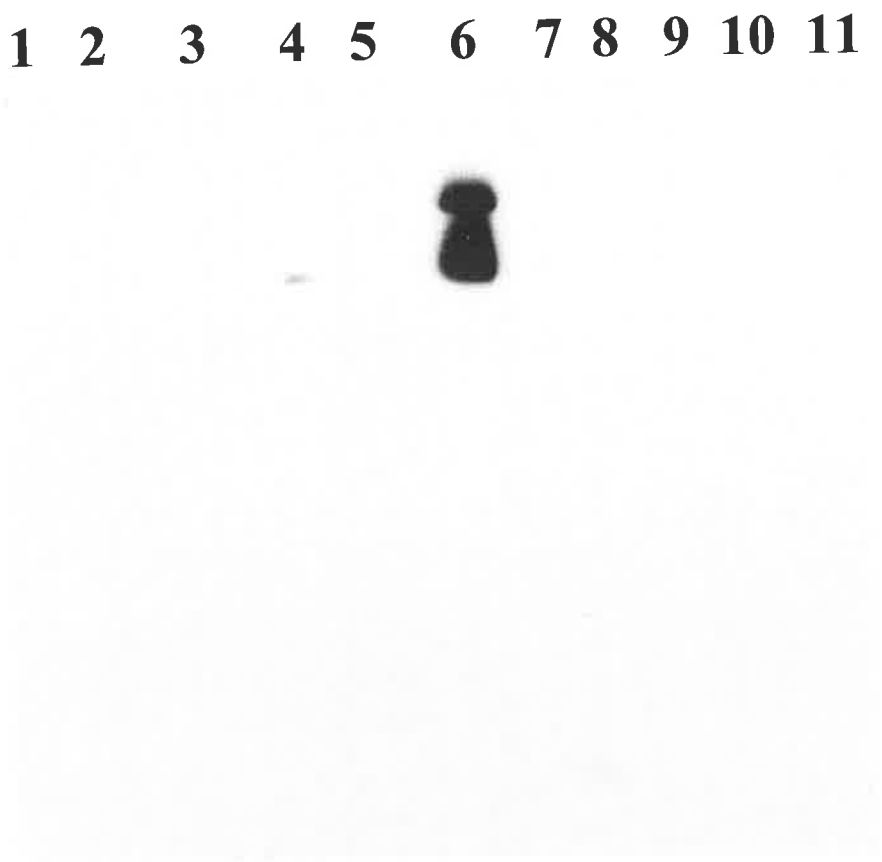
Fig. 6.18 Western blot analysis of hybrid CstH::LT-B_{44-64} proteins expressed by recombinant *cst* operons.

The presence of the foreign epitope in hybrid proteins expressed from recombinant *cst* operons in different plasmid vectors was analysed by Western blot, using Mab #39 against LT-B (Panel A) and polyclonal antiserum against CT (Panel B) and detected by ECL. Whole cell lysates of 10^9 cells were electrophoresed in SDS on 15% polyacrylamide gels, transferred to a nitrocellulose filter and probed with the antibody. The arrow indicates the position of the LT-B.

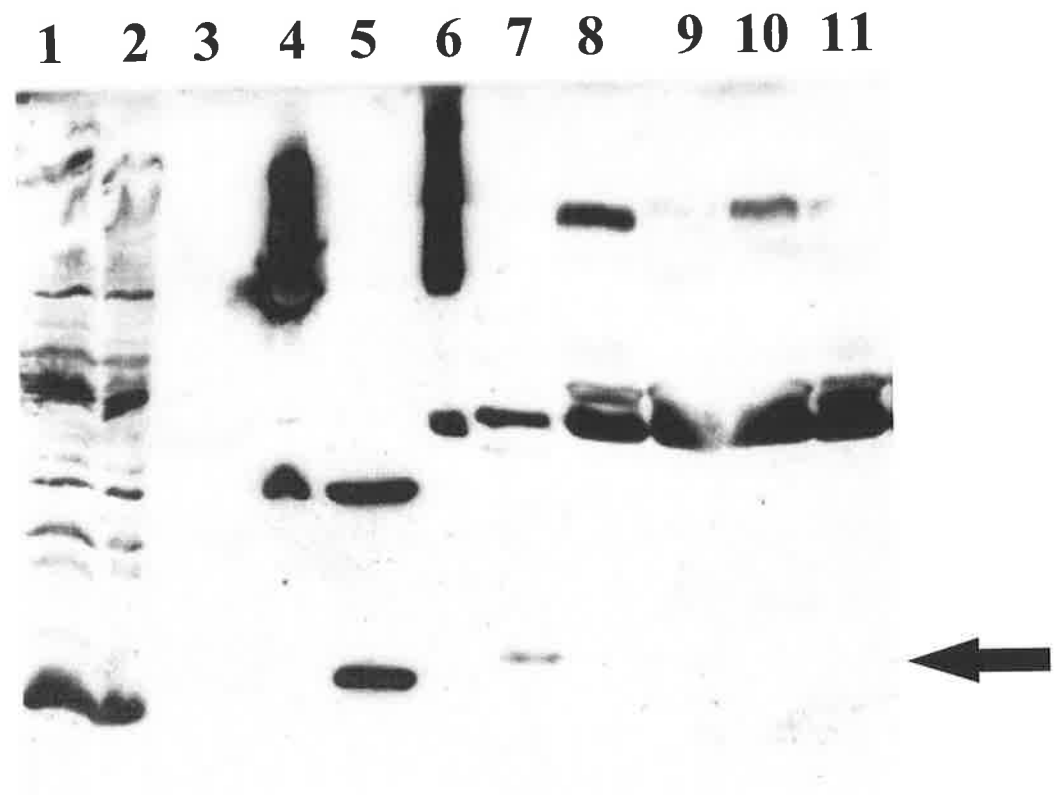
Lanes:

1. PB176 (+control)
2. PB176P$^-$(-control)
3. *E. coli* K-12[pBluescript] vector plasmid for *cstH::elt-*B_{44-64} (-control)
4. Non-denatured CT(+control)
5. Denatured CT(+control)
6. Non-denatured LT(+control)
7. Denatured LT (+control)
8. *E. coli* K-12[pPM4588] expressing CstH::LT-B_{44-64} (between aa 65-66), pBluescript vector
9. *E. coli* K-12[pPM4593] expressing CstH::LT-B_{44-64} (between aa 99-100), Pbluescript vector
10. *E. coli* K-12[pPM4713] expressing CstH::LT-B_{44-64} (between aa 65-66), pBC KS vector
11. *E. coli* K-12[pPM4715] expressing CstH::LT-B_{44-64} (between aa 99-100), pBC KS vector

A



B



fragments encoding *cst* operons from plasmids pPM484, pPM4585 and pPM4593 were ligated into the *Hin*dIII site of pBC KS to give plasmids pPM4568, pPM4713 and pPM4715, respectively (Fig. 6.19). The ligations were transformed into *E. coli* K-12 strain DH5α and transformants were selected for Cm^R and screened by restriction digestion analysis. Clones resistant to Cm and containing plasmids of the correct size were selected for further investigation. The orientation of the cloned DNA in the plasmid vector (T7 or T3 promoters) was confirmed by restriction enzyme digestion of the constructs. Bacteria harbouring plasmids containing the hybrid *cst* operon in the T3 promoter orientation were used for expression of the hybrid CstH proteins.

Western blot analysis was employed to assess the expression of the recombinant CstH through the pBC KS vector plasmid using a polyclonal antiserum and Mab CH10 against CS3. This revealed a band with a higher molecular weight than CstH corresponding to the hybrid CstH (Fig 6.20). The hybrid proteins could also be recognized by polyclonal antisera against CT but not by Mab against LT, Mab 39 (Fig 6.18) as described in section 6.2.6.2.

Comparison of the expression of the CstH hybrid proteins from pBluescript and pBC KS indicated that pBC KS expressed hybrid proteins at a higher level than pBluescript, especially the hybrid with the insertion between amino acids 99 and 100, (Fig 6.21) suggesting that this vector may be better for the expression of the hybrid proteins.

6.3 Discussion

In this chapter, a first stage in the development of the CS3 fibrillar adhesin system for delivery of foreign antigenic determinants has been described. This study suggested the potential of CstH for insertion and expression of foreign antigenic

Fig 6.19 Re-cloning of recombinant *cst* operons into pBC KS

Plasmids pBC KS, pPM484, pPM4588 and pPM4593 were digested with *Hin*dIII and the DNA fragments containing wild type or recombinant *cst* (r*cst*) operons were cloned into the *Hin*dIII site of pBC KS. The plasmids containing *cst* and r*cst* operons in T3 orientation were selected and designated pPM4568, pPM4713 and pPM4715, respectively.

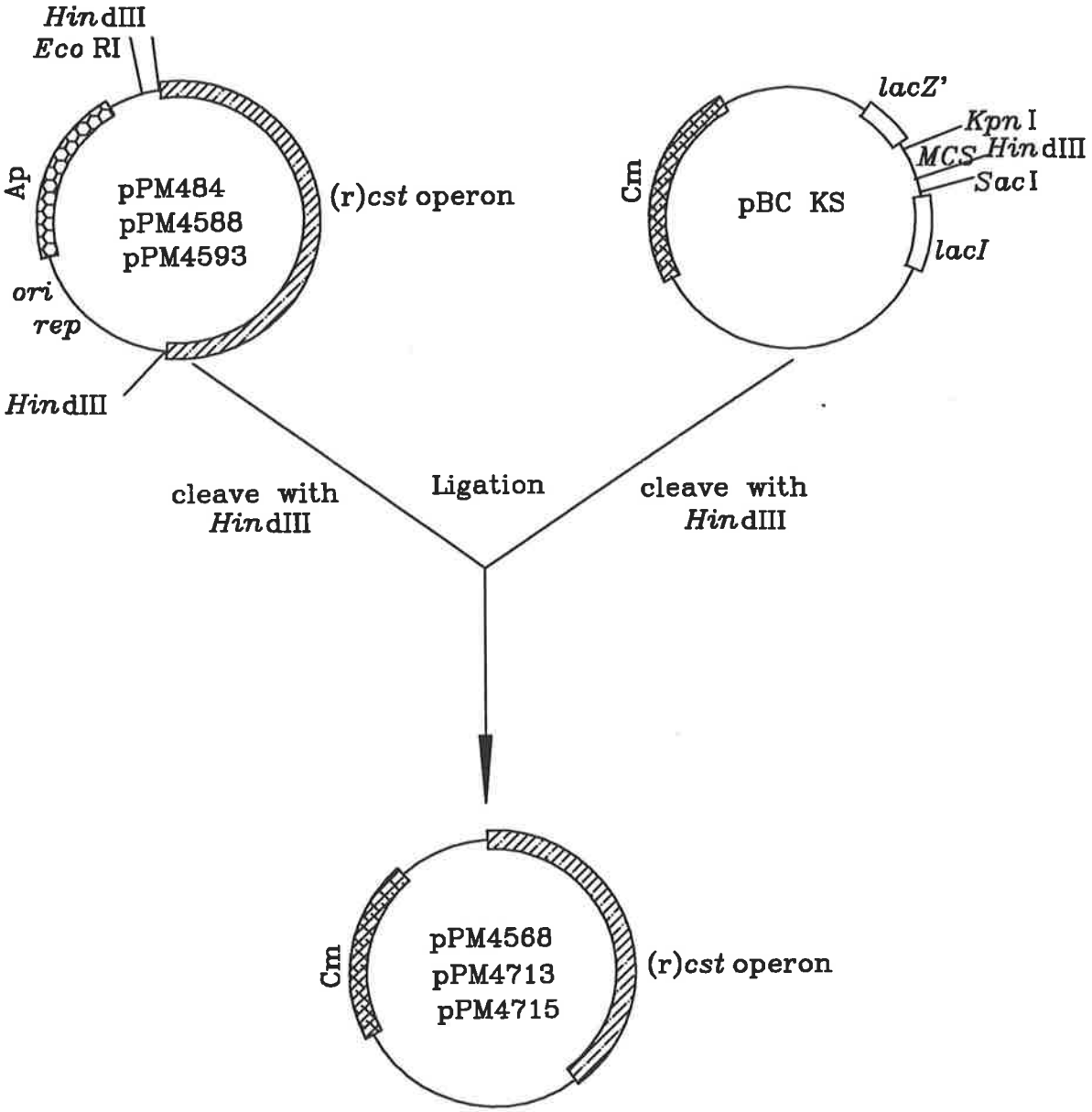

*Hin*dIII
Eco RI
Ap
pPM484
pPM4588
pPM4593
(r)*cst* operon
ori
rep
*Hin*dIII
Cm
pBC KS
lacZ'
Kpn I
MCS
*Hin*dIII
*Sac*I
lacI
cleave with
*Hin*dIII
Ligation
cleave with
*Hin*dIII
Cm
pPM4568
pPM4713
pPM4715
(r)*cst* operon

Fig. 6.20 Western blot analysis of the hybrid CstH proteins expressed by recombinant *cst* operons in pBC KS.

The CstH::LT-B_{44-64} hybrid proteins were expressed from recombinant *cst* operons, analysed by Western blot using appropriate antibodies and detected by ECL. Whole cell lysates were electrophoresed in SDS on 15% polyacrylamide gels, transferred to a nitrocellulose filter and probed with either a polyclonal antisera to CS3 (Panel A) or Mab CH10 to CstH (Panel B). The arrow indicates the position of CstH.

Lanes:

1. PB176 (+control)
2. *E. coli* K-12[pBC KS] vector plasmid for *cstH::elt-*B_{44-64} (-control)
3. *E. coli* K-12[pPM484] expressing wild type CstH
4. *E. coli* K-12[pPM4713] expressing CstH::LT-B_{44-64} (between aa 65-66)
5. *E. coli* K-12[pPM4715] expressing CstH::LT-B_{44-64} (between aa 99-100)

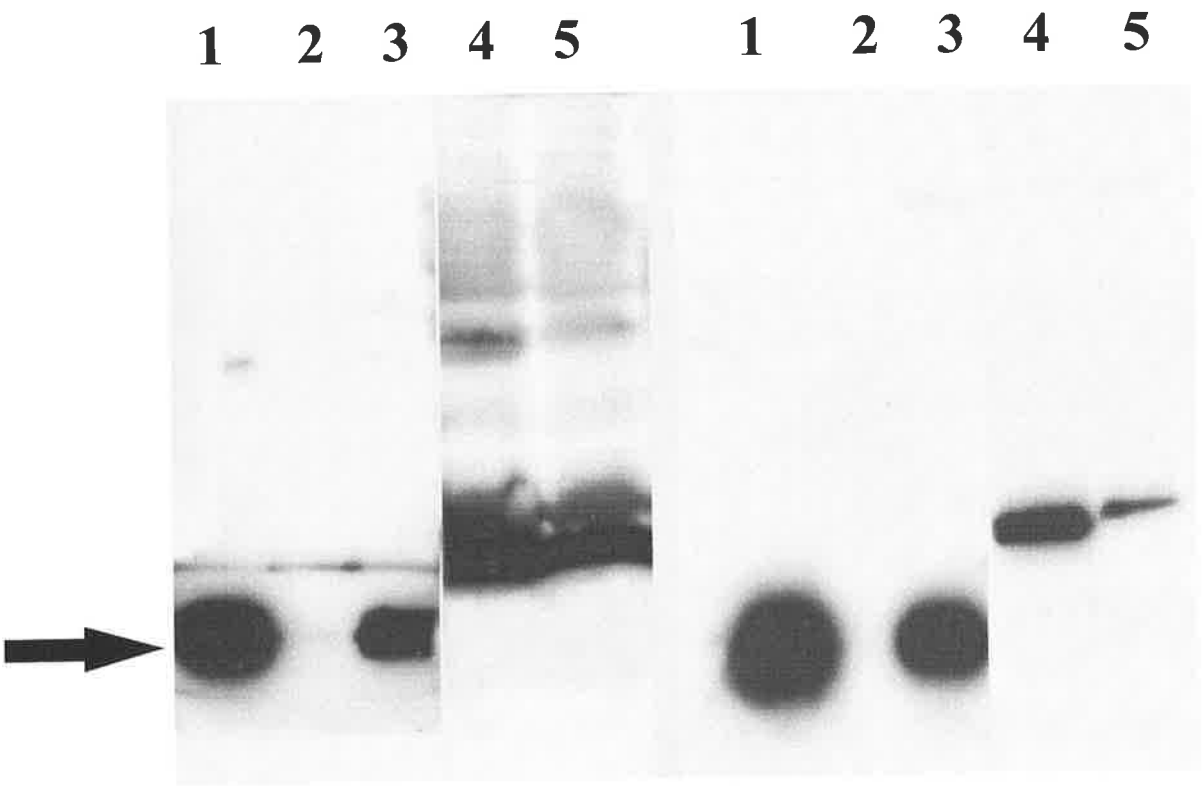
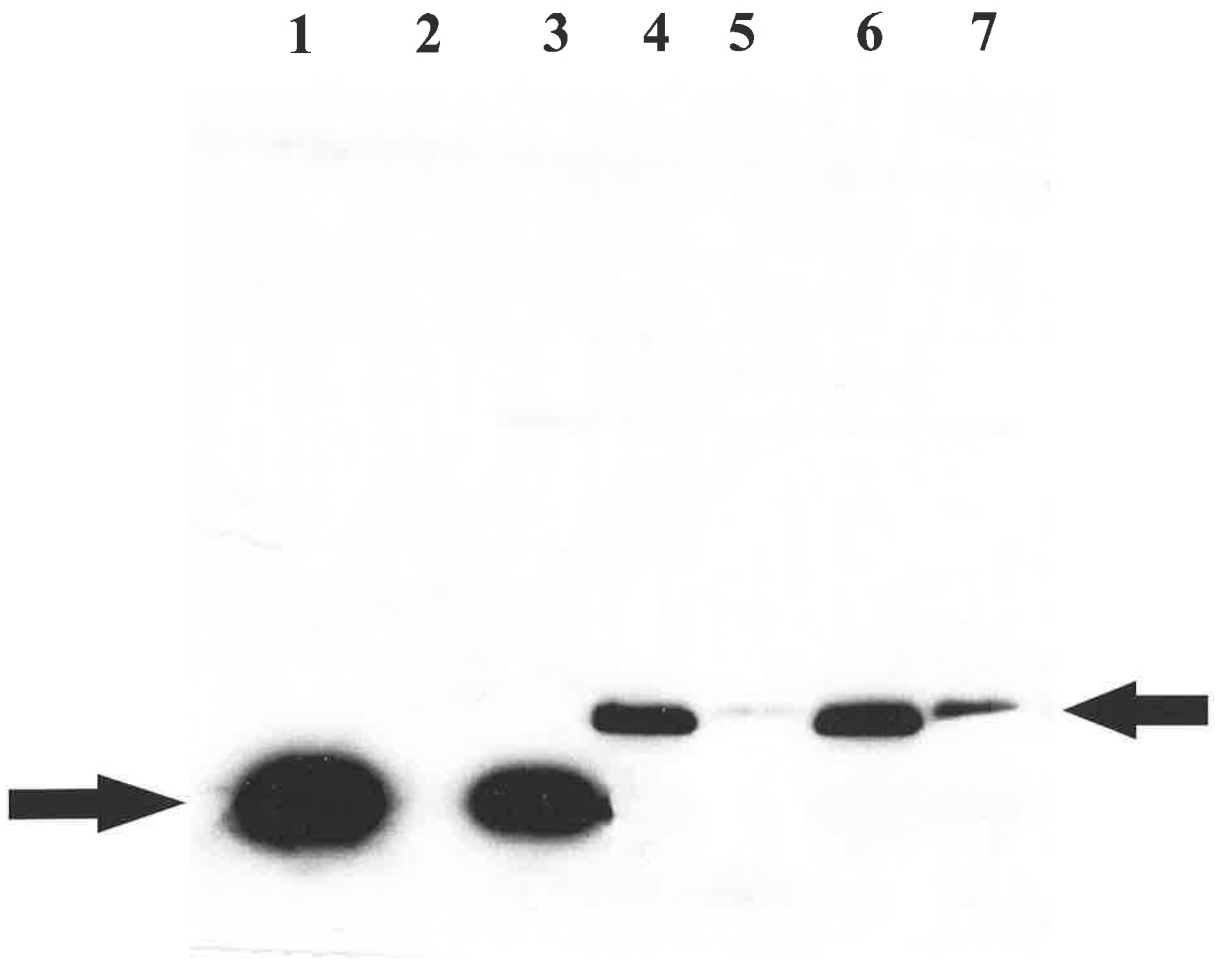

1 2 3 4 5
1 2 3 4 5
A
B

Fig. 6.21 Comparison of the expression of the CstH::LT-B_{44-64} proteins from different vectors

The expression of the CstH::LT-B_{44-64} hybrid proteins from recombinant *cst* operons in different plasmid vectors was analysed by Western blot using Mab CH10 to CstH and detected by ECL. Whole cell lysates of 10^9 cells were electrophoresed in SDS on 15% polyacrylamide gels, transferred to a nitrocellulose filter and probed with the antibody. The left arrow indicates the position of the CstH and the right arrow hybrid CstH.

Lanes:

1. PB176 (+control)
2. *E. coli* K-12[pBluescript] vector plasmid for *cstH::elt-*B_{44-64} (-control)
3. *E. coli* K-12[pPM484] expressing wild type CstH
4. *E. coli* K-12[pPM4588] expressing CstH::LT-B_{44-64} (between aa 65-66); vector pBluescript
5. *E. coli* K-12[pPM4593] expressing CstH::LT-B_{44-64} (between aa 99-100); vector pBluescript
6. *E. coli* K-12[pPM4713] expressing CstH::LT-B_{44-64} (between aa 65-66); vector pBC KS
7. *E. coli* K-12[pPM4715] expressing CstH::LT-B_{44-64} (between aa 99-100); vector pBCKS


1 2 3 4 5 6 7

determinants. The major subunit of the fimbriae may be suitable as a carrier of heterologous antigenic determinants if insertion of such epitopes into the protein does not distort the structure of the subunit, but allows the epitope to be expressed on the hybrid fimbriae.

It has been demonstrated that the highly variable domains of P-fimbriae, K88 and CS31A are suitable areas for insertion or replacement by foreign epitopes (Van Die *et al.,* 1990; Van der Zee *et al.,* 1995, Bakker *et al.,* 1990; Thiry *et al.,* 1989; and Bousquet *et al.,* 1994). The exposed area or epitopes of a protein are also thought to be good candidates for insertion of foreign antigenic determinants. However there are no variable domains in CstH (Chapter 4) suitable for insertion or replacement with heterologous peptides. Since it was not possible to define the epitopes of CstH by experimental approaches (Chapter 5), three potentially exposed areas of CstH were chosen for insertion sites, based on computer analysis of the secondary structure of the protein.

The introduction of restriction sites into three predicted epitopes of CstH resulted in the substitution of amino acids #66 and 67 (Ser_{66}→Leu and Lys_{67}→Glu), amino acid #100 (Ala_{100}→Leu) and #109 (Asn_{109}→Leu) of the mature protein in the three mutants, respectively. Experimental analysis of the mutants by immunoblot using polyclonal and two monoclonal antibodies showed that the mutants reacted with all of the antibodies.

As the foreign epitope, a peptide corresponding to residues 44 to 64 of hLT-B, containing a continuous epitope near the GM_1-binding domain of the B subunit (Finkelstein, 1987; Kazemi and Finkelstein, 1991), was chosen on the basis that it is a conserved sequence between CT-B and LT-B. Several peptides corresponding to this sequence or part of it have been used for immunization and the antibodies that were

generated showed cross-reactivity with CT-B and LT-B, and in some cases neutralized these toxins (Jacob *et al.,* 1983, 1984, 1986; Pedoussout *et al.,* 1989; Guyon-Gruaz *et al.,* 1986; Ghose and Karush, 1988). It was assumed that this peptide would be appropriate for use as a linear epitope to evaluate the potential of CstH as a carrier. The hybrid proteins could then be assessed to see if they could be assembled as chimeric pili capable of eliciting an antibody response against both determinants. Such hybrid pili may be useful as components of a multivalent vaccine against diarrhoeal diseases. Computer analysis of the hybrid proteins also suggested the exposure of the inserted hLT-B epitope.

Expression of the hybrid proteins was monitored in different expression systems by immunoblot using antibodies against native CS3, CT and LT-B. Expression of hybrid proteins from their natural promoters was unsuccessful even in an *E. coli* deg^p strain (defective in the degradation of periplasmic proteins) and in the presence of the assembly genes, in trans. Presumably, the production of protein is so low that it is not detectable by immunoblot analysis. When the T7 promoter/RNA polymerase was used, CstH::LT-$B_{44\text{-}64}$ hybrid proteins were detected with polyclonal and monoclonal antibodies against CS3, although a polyclonal antibody against native CT and an Mab to native LT-B could not recognize the hybrid proteins. The Mab to LT-B was against the B-pentamer (Gunhild Johnson personal communication), so it is likely that this Mab does not react with the specific epitope of LT-B.

The hybrid proteins were expressed from the native CS3 biosynthetic operon by replacing the wild type *cstH* gene with the hybrid genes in the *cst* operon. The hybrid proteins could be detected by using polyclonal antisera against CS3 and CT and Mab against CS3. However, the expression of the hybrid proteins was low, because a large number of cells (more than 10^9) was needed for detection. The expression of the hybrid

proteins was lower than for the native CstH and the production of hybrid proteins with an insertion between amino acids 99 and 100 was very poor.

Re-cloning of hybrid *cst* operon into pBC KS improved the expression of the hybrid proteins as shown by Western blot using equal numbers of cells harbouring plasmids pBluescript or pBC KS encoding the same hybrid proteins.

The poor level of expression of the hybrid pili may simply be a direct reflection of plasmid loss. In Ap-containing cultures, bacteria harbouring plasmids with a β-lactamase gene release the enzyme into the medium and Ap will be degraded allowing growth of plasmid free cells. Bacteria expressing the hybrid proteins seem to be more susceptible to plasmid loss. Thus, bacteria lacking the plasmid encoding hybrid CstH are likely to overgrow those bacteria with plasmids encoding the hybrid protein. In such cultures where cells lose their plasmids, there are insufficient cells still producing protein for it to be detected by antibody by Western blot analysis. However, pBC KS-based plasmids have the advantage that the Cm does not degrade in the culture and bacteria without plasmid do not overgrow and thus this system is better for the expression of the hybrid proteins

In conclusion, although expression of hybrid CstH::LT-B_{44-64}can be problematic, the results indicate that the CstH can accept insertion of heterologous epitopes in at least two sites, and has the potential to be used as a carrier for the expression of foreign antigenic determinants.

Chapter 7

Construction of CstH::ST hybrid protein

7.1 Introduction

In the previous chapter, the construction of CstH::LT-B_{44-64} hybrid proteins was described. The results showed that CstH can tolerate the insertion of a 20-aa peptide. To test the effect of the insertion of a complex peptide into the CstH protein it was decided that the mature form of ST, which contains 3 disulfide bonds, would be used.

Two types of ST are involved in the pathogenesis of ETEC. Of particular interest here is STa of which there are several structural forms produced by different enteric bacteria. The STa family of toxins are either 18 or 19 aa extracellular peptides which result from two proteolytic cleavages from a 72 aa precursor (Rasheed *et al.,* 1990). They have a common highly conserved region of 10 aa, and 6 conserved cysteine residues which form three disulfide bonds (Shimonishi *et al.,* 1987). The tertiary structure formed by these bonds is critical for the expression of the biological activities of STa.

Monoclonal antibodies (MAb) against STa have been produced and used for diagnosis and epitope mapping of the peptide (Brandwein *et al.,* 1985; Svennerholm, 1986; Takeda, 1993). Three distinct antigenic determinants have been recognized in STh using MAbs against the native STh (Takeda *et al.,* 1993). These studies demonstrated that the intact native structure of the peptide is required for proper MAb binding.

ST toxin is a low molecular weight, non-immunogenic peptide toxin but it has been shown that ST is haptenic and can become immunogenic when coupled to a large molecular weight carrier. Several different carrier molecules have been used to prepare immunogenic toxoids of STa including: outer membrane protein OmpC (Saarilahti *et al.,* 1989), bovine serum albumin (Frantz and Robertson, 1981), LT-A (Sanchez *et al.,* 1986), LT-B (Clements, 1990, 1993) and IgG binding fragment of *Staphylococcus aureus* proteinase A (Lowenadler *et al.,* 1991).

Native and synthetic STa have been used to construct fusion proteins with different carriers through chemical procedures (Klipstein *et al.,* 1982; 1984) and recombinant DNA technology (Aitken and Hirst, 1993; Clements, 1990; Houghten *et al.,* 1985; Sanchez *et al.,* 1988). These fusion proteins have been used to elicit antibody responses and recently positive results were obtained (Cardenas and Clements, 1993) using LT-B::ST fusions which mediated mucosal protection against both ST and LT by oral immunization.

In this chapter, the construction of the CstH::ST fusion proteins will be described. In order to express ST with CstH, DNA encoding mature ST has been cloned in-frame within *cstH* and the fusion proteins examined with antibodies directed against CS3 and ST.

7.2 Results

7.2.1 Construction of an *est* gene cassette

For expression of ST within CS3, a DNA fragment encoding the mature ST was generated for insertion into mutant *cstH* genes. This fragment was prepared from the *est* gene isolated from a ST^+ ETEC strain, PB176.

7.2.1.1 Construction of a cosmid gene bank from PB176 plasmid DNA

Purified plasmid DNA from PB176 was prepared using CsCl isopycnic gradient centrifugation. The DNA was partially digested with *Sau*3AI and ligated into the *Bam*HI site of the mobilizable cosmid vector pPM2101 (Sharma *et al.,* 1989). After *in vitro* packaging, the cosmids were transduced into *E. coli* K-12 strain DH1 and the transformants were screened for the *est* gene using a Dig-labelled *est*-specific DNA probe (oligo # 299) based on the known sequence of *est* (Moseley *et al.,* 1983). Three positive clones, pPM793, pPM801 and pPM807 were characterized further by Southern hybridisation using the same probe and purified DNA from these clones digested with *Hin*dIII. Hybridisation analysis showed that the *est* gene was present on a *Hin*dIII fragment of >8 kb (Fig. 7.1). This fragment was purified from pPM793 and cloned into the *Hin*dIII site of pBluescript SK and transformants were screened for *est* as above. One positive clone designated pPM4564 was selected and used for further work.

7.2.1.2 Subcloning of the *est* gene

The est^+ clone, pPM4564, was used as a source of DNA for subcloning the *est* gene. Southern hybridisation analysis identified a 1.9 kb *Bam*HI fragment containing *est* (Fig. 7.2). This fragment was purified and cloned into the *Bam*HI site of pBluescriptSK to give plasmid pPM4565 (Fig. 7.3), which was confirmed by sequencing. The fragment contained the full-length *est* gene (360 bp) encoding the 72 aa pre-pro-ST protein (Fig. 7.4 A). This fragment was identical with the *estA*3 gene (Moseley *et al.*, 1983). This clone, which was designated pPM4565 was used for the construction of the *est* gene cassette.

Fig. 7.1 Southern analysis of DNA of cosmids pPM793, pPM801 and pPM807

DNA was digested with *Hin*dIII and electrophoresed on a 1% agarose gel. Plasmid DNA from PB176 and pPM2101 were used as positive and negative controls respectively. After transfer to nitrocellulose the filter was probed with Dig-labelled oligo #299. The position of the DNA fragment containing *est* is shown by an arrow.

Lanes:

1. PB176
2. pPM2101
3. pPM793
4. pPM801
5. pPM807

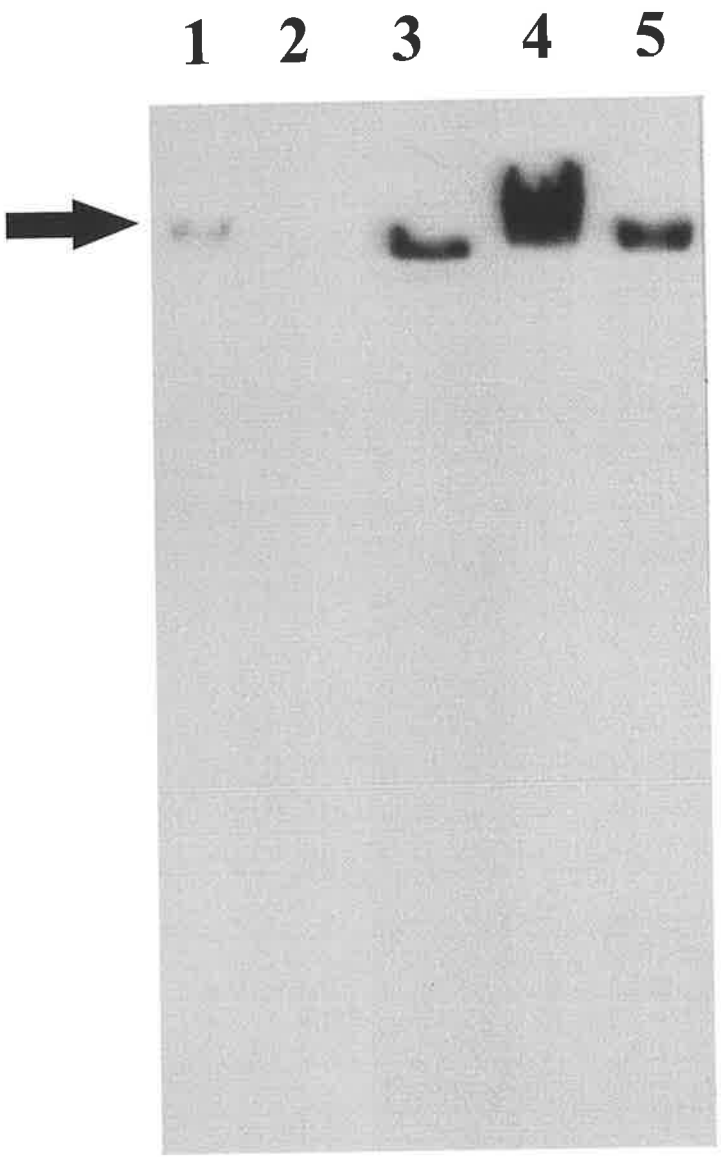

1
2
3
4
5

Fig. 7.2 Southern analysis of *est* positive clones

DNA was digested with restriction enzymes and electrophoresed on a 1% agarose gel. After transfer to nitrocellulose the filter was probed with Dig-labelled oligo #299. A 1.9 kb *Bam*HI fragment containing the *est* gene is indicated by the arrow. SPP1 phage DNA digested with *Eco*RI was used as a size standard on the ethidium bromide stained agarose gel (not shown).

Lanes:

1. Whole PB176 DNA digested with *Hin*dIII (+control)
2. pBluescript SK digested with *Hin*dIII (-control)
3. pPM4564 digested with *Hin*dIII and *Eco*RI
4. pPM793 digested with *Hin*dIII
5. pPM2101 digested with *Hin*dIII (-control)
6. pPM793 digested with *Hin*dIII and *Eco*RI
7. pPM4564 digested with *Bam*HI

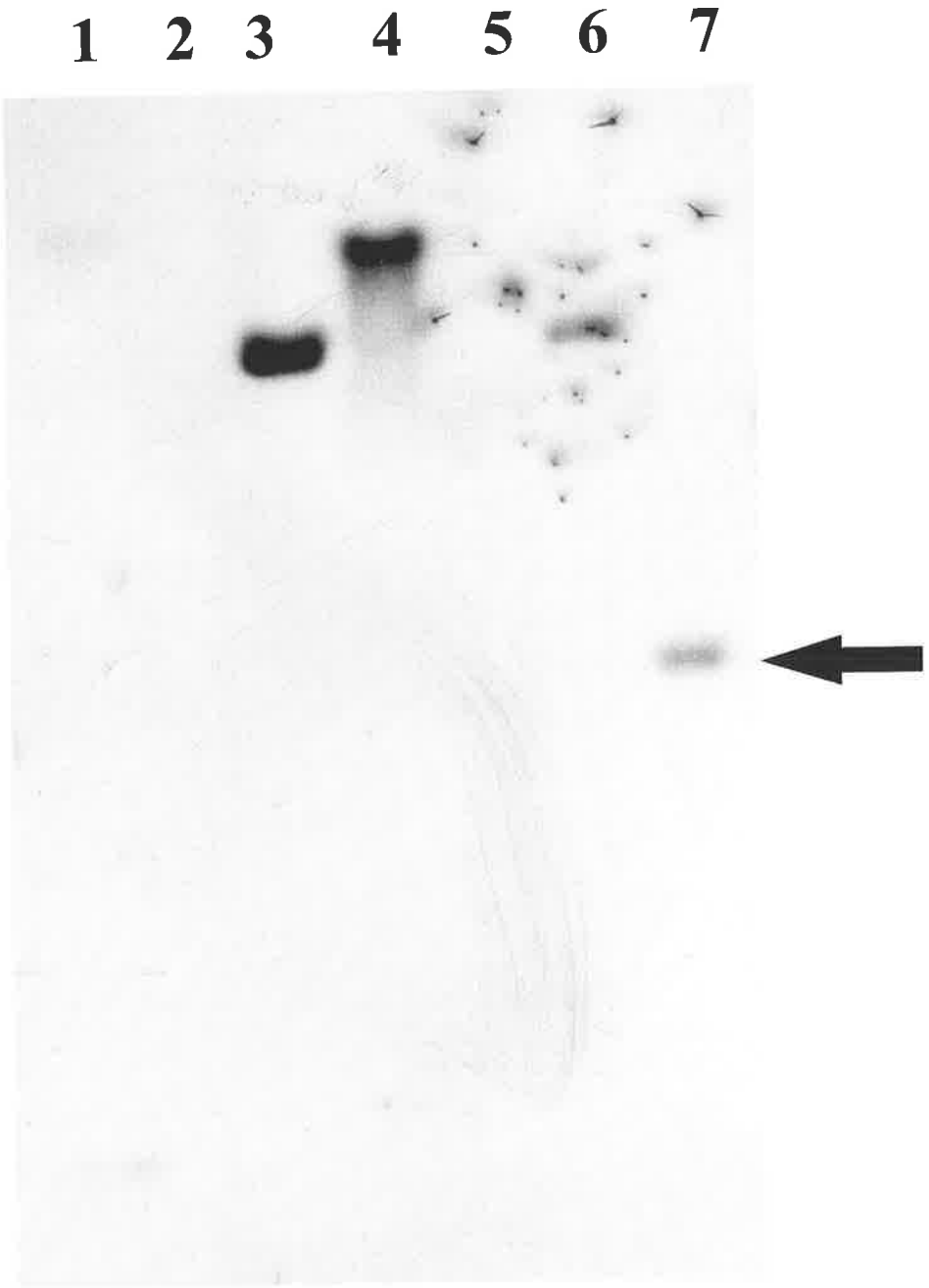

1 2 3 4 5 6 7

Fig. 7 3 Construction of plasmid pPM4565.

Plasmid DNA from ETEC strain PB176 was partially digested with *Sau*3AI and cloned into the *Bam*HI site of pPM2101. Transformants were selected as Ap^R and positive in DNA hybridization using Dig-labelled oligo #299. DNA from a positive plasmid, called pPM793, was digested with *Hin*dIII and a > 8 kb fragment cloned into pBluescript to produce pPM4564. pPM4564 digested with *Bam*HI and a 1.9 kb fragment ligated into the *Bam*HI site of pBluescript SK to give plasmid pPM4565.

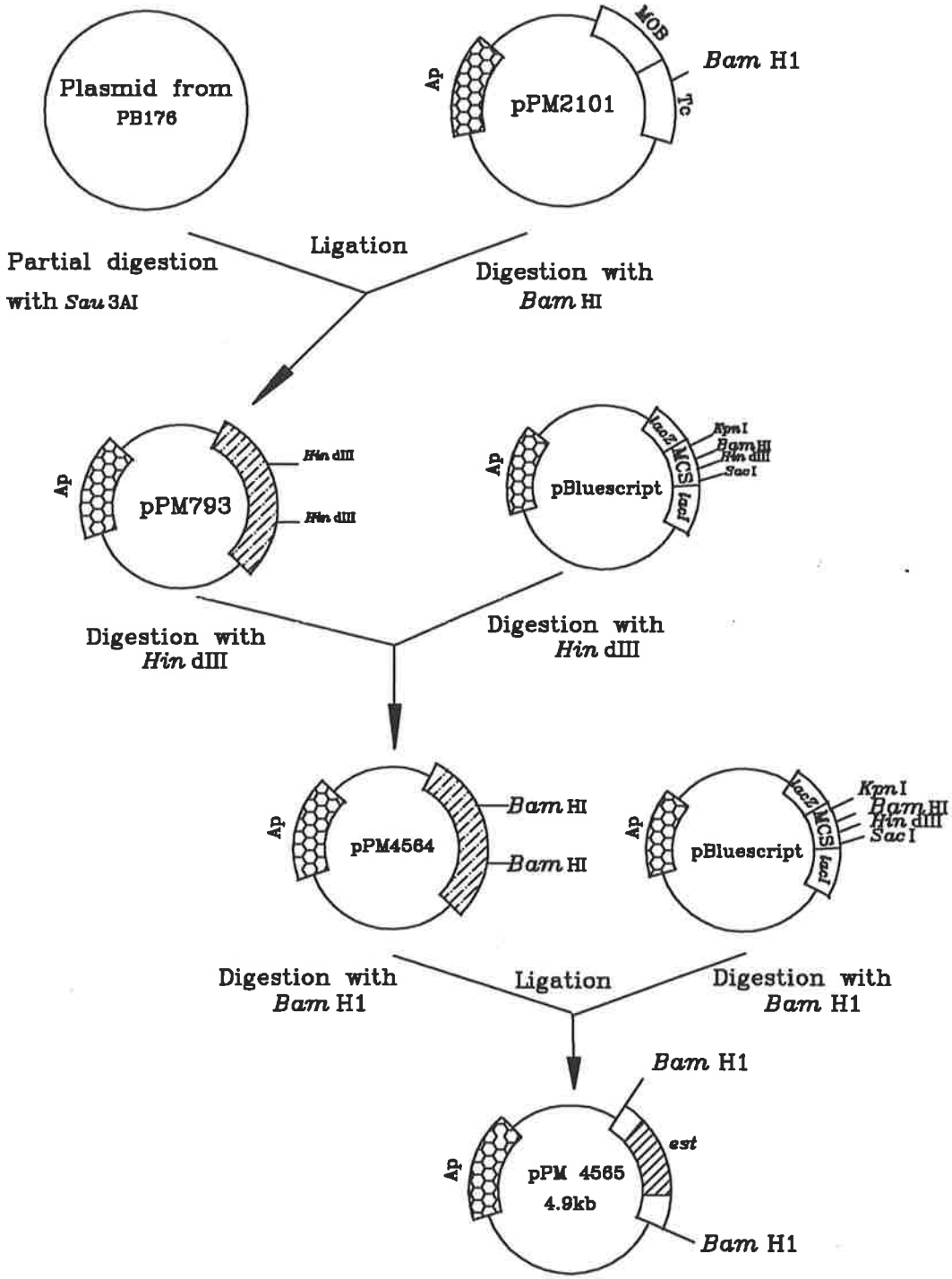

Plasmid from
PB176
pPM2101
MOB
Tc
Ap
Bam H1
Ligation
Partial digestion
with Sau 3AI
Digestion with
Bam HI
pPM793
Hin dIII
Hin dIII
Ap
pBluescript
lacZ
MCS
lacI
Kpn I
Bam HI
Hin dIII
Sac I
Digestion with
Hin dIII
Digestion with
Hin dIII
pPM4564
Bam HI
Bam HI
Ap
pBluescript
Kpn I
Bam HI
Hin dIII
Sac I
Digestion with
Bam H1
Ligation
Digestion with
Bam H1
Bam H1
est
pPM 4565
4.9kb
Ap
Bam H1

Fig. 7.4 The nucleotide sequence of the 1794 bp fragment containing the *est* gene

A

The sequence of the 1794 bp DNA fragment containing *est* gene cloned from PB176 is presented. Underlined sequence between nt 476 and 835 encodes pre-pro-ST which is identical to *estA3* and *estA4* (Mosely et al., 1983; Staieglitz et al., 1988). Translation of the region encoding pre-pro-ST has been indicated. Termination site is shown by stars

B

The homology of the DNA with published sequences has been defined using BLASTN program

C

1.9 kb DNA fragment with relevant restriction sites has been shown. boxes show ORFs.

A

```
1      GAA TTC CTG CAG CCC GGG GGA TCC AAC GCC GCA GAT TTC TTT TCG AAG    48
49     GGT AGC CCA ATT CGC GTA CGA CGG GAG CCA GCT TCT TAC CAT AAT TTG    96
97     AAA ATA AAG CTC AAT GGT GCG GAT TTT TTC TTC TTC AGT AAA CAT GTT   144
145    TCA TCT CCT GAG AGT CTA GGA AAT CGT CCG CAT CCC CCT TCC GCA ACT   192
193    TGG GTG AAG ATG TTC ATA GGC ACA TCT ACG GTG GCA TGT CCG GTA ATG   240
241    GGG TAA ATG ATA ATA GGT GTT GGG GAA ATC CAA CTG CAT TAA GTA TGC   288
289    ATT GTA ATT TTT TTG TGA TTA ACC CCA CAA AAA CAG TCA TTA AAC ATA   336
337    AAT ATA TCA TTT TGC TTA AAC AAC CTT TCC TGT TTT TTT CGG GTC GCC   384
385    GAA AAA GAT AAT ATT ACT ATG GCT CTT CGT AGG CGG AGA GTA TAG TAT   432
433    GAT GTT CAT CCA CCA AAA AAA TAA AAA AAA GTT TGC GCA ATC GTT CTG   480
1                                                          Met Lys      2
481    GTT TTG ATT CAA ATG TTC GTG GAT GCC ATG TCC GGA GGT AAT ATG AAG   528

3      Lys Ser Ile Leu Phe Ile Phe Leu Ser Val Leu Ser Phe Ser Pro Phe    18
529    AAA TCA ATA TTA TTT ATT TTT CTT TCT GTA TTG TCT TTT TCA CCT TTC   576

19     Pro Gln Asp Ala Lys Pro Val Glu Ser Ser Lys Glu Lys Ile Thr Leu    34
577    CCT CAG GAT GCT AAA CCA GTA GAG TCT TCA AAA GAA AAA ATC ACA CTA   624

35     Glu Ser Lys Lys Cys Asn Ile Ala Lys Lys Ser Asn Lys Ser Gly Pro    50
625    GAA TCA AAA AAA TGT AAC ATT GCA AAA AAA AGT AAT AAA AGT GGT CCT   672

51     Glu Ser Met Asn Ser Ser Asn Tyr Cys Cys Glu Leu Cys Cys Asn Pro    66
673    GAA AGC ATG AAT AGT AGC AAT TAC TGC TGT GAA TTG TGT TGT AAT CCT   720

67     Ala Cys Thr Gly Cys Tyr ***                                        72
721    GCT TGT ACC GGG TGC TAT TAA TAA TAT AAA GGG AAC TAA ACA GTT CCC   768
769    TTT ATA TTT GTT CTG ATT CTG ATG ATG TCT GTA ACG TAT GTA CCT GTT   816
817    GCT TTG TTG AAT AAA TCG AAC TTT CGC TGG GTT GAA GGA GCA GAG CAC   864
865    GCA TCA TCC GGC AAC ATG AGT CGT TCC ATG GTA AAG CAG AAG TTC AGA   912
913    ATC ACC AAC TGG CGC AGC CAC AAC AAA GCC CTT ATC CTC CGT GGC ACC   960
961    ATC ACT TTC TGG CGG GAT GGC GAG GCA ATC CAG GCC TGG TAT GAG TCA  1008
1009   GCA ACC CCC TCA TCA CGG GGA CGA CCT CAG AGT TAT TCT GAC CTT GCT  1056
1057   ATT ACC ACT GCC CTT GTG ATT AAA CGC GTA TTC CCG GCT GAC CCC ACC  1104
1105   CTG CGG GCT TGA ACT GCC CCC GAA AGT TGG ACA AGC TAT TTG TTA GAC  1152
1153   GGC TAG CTG TGC CTG AGT CCG GTA TTC TAT CGG GCT CAG GCC ATT TAA  1200
1201   CCT TGA GCT AAT CCG CTC ATT GTT AAT CGA TAT ACT CCT CGA CTA CTC  1248
1249   TTC GAC GCT CTT TTG TAC TAT AAT AAG CTT TTA CTG CTT CAG CCA GGA  1296
1297   CTC CAT ACC GCG CTG ATG CCT GCC GTT CCA TTC CTG CTG TGT AAG CGT  1344
1345   CAA CGG AGC ACC GCA TTG ACG CTT ATT TAT TGG TGA GTA CTA CGT TCC  1392
1393   ATG GCA GGA GTT CGC CAA CTC GGT TGG AAG GCA TTC CGG CAG TAC GCT  1440
1441   CAG AAT ATG GGC AGA TAC GCT TCC GGA TCG ATA CCG TTC AGA CGG GAG  1488
1489   GTG CCG ATC AGC CCG TAC AGA GGG GTC CAC GGT TCG CCG CCG TGA TCG  1536
1537   GTA TCG CTA CCC AAG GAA AAC GTA GGA TTG GGA TTG ACC TTT ATA TAC  1584
1585   TCA CTA TAG CAT TGG TGG GAT ATT CCA GAG GAG GTC ATT ATG CAT TCG  1632
1633   GAA AAT ATT GCA GCT TAC ATC GGG CTG GAT GTA CAT AAA GAA ACG TTG  1680
1681   GCG GTT G?T ATT GCT GCA CCT GAA CGG CTT GGC GAA GTT CGT TAT TAT  1728
1729   GGT ACG ATT AAC AAC GAA GCC CAG GCT GTA CGT CGA TTA TTT CAG AAA  1776
1777   TTG CAG GGG ACT TTA CGG                                        1794
```

Transposon Tn*1681* encoding heat-stable (st) toxin.Identities = 196/297 (65%), Strand = Plus / Plus

```
pPM4565  448 AAAAAATAAAAAAAAGTTTGCGCAATCGTTCTGGTTTTGATTCAAATGTTCGTGGATGCC 507
             |||||| || |||||  ||||  ||||  | |   |     |  ||          |   |
Tn1681   211 AAAAAAAAATAAAAAAATTGCAAAATCCGTTTAACTAATCTCAAATATCCGTGAAACAAC 270
pPM4565  508 ATGTCCGGAGGTAATATGAAGAAATCAATATTATTTATTTTTCTTTCTGTATTGTCTTTT 567
             ||| | |||||||| |||||  ||    ||| ||     |||||| |||||||||| |||||
Tn1681   271 ATGACGGGAGGTAACATGAAAAAGCTAATGTTGGCAATTTTTATTTCTGTATTATCTTTC 330
pPM4565  568 TCACCTTTCCCTCAGGATGCTAAACCAGTAGAGTCTTCAAAAGAAAAAATCACACTAGAA 627
              |  ||||   ||||    || || || | || ||||||||||| |||||  ||| ||||
Tn1681   331 CCCTCTTTTAGTCAGTCAACTGAATCACTTGACTCTTCAAAAGAGAAAATTACATTAGAG 390
pPM4565  628 TCAAAAAAATGTAACATTGCAAAAAAAAGTAATAAAAGTGGTCCTGAAAGCATGAATAGT 687
              | ||||| ||| |  ||| ||||||  |  | | |||       | ||||  ||||| |
Tn1681   391 ACTAAAAAGTGTGATGTTGTAAAAAACAACAGTGAAAAAAAATCAGAAAATATGAACAAC 450
pPM4565  688 AGCAATTACTGCTGTGAATTGTGTTGTAATCCTGCTTGTACCGGGTGCTATTAATAA 744
             |    ||||||||||||| | |||||||||||||| ||| | || || ||||||  ||
Tn1681   451 ACATTTTACTGCTGTGAACTTTGTTGTAATCCTGCCTGTGCTGGATGTTATTAAAAA 507
```

***E.coli* heat-stable enterotoxin gene, complete cds.Identities = 360/360 (100%), Strand =Plus / Plus**

```
pPM4565  476 TTCTGGTTTTGATTCAAATGTTCGTGGATGCCATGTCCGGAGGTAATATGAAGAAATCAA 535
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
estA3      1 TTCTGGTTTTGATTCAAATGTTCGTGGATGCCATGTCCGGAGGTAATATGAAGAAATCAA 60
pPM4565  536 TATTATTTATTTTTCTTTCTGTATTGTCTTTTTCACCTTTCCCTCAGGATGCTAAACCAG 595
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
estA3:    61 TATTATTTATTTTTCTTTCTGTATTGTCTTTTTCACCTTTCCCTCAGGATGCTAAACCAG 120
pPM4565  596 TAGAGTCTTCAAAAGAAAAAATCACACTAGAATCAAAAAAATGTAACATTGCAAAAAAAA 655
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
estA3:   121 TAGAGTCTTCAAAAGAAAAAATCACACTAGAATCAAAAAAATGTAACATTGCAAAAAAAA 180
pPM4565  656 GTAATAAAAGTGGTCCTGAAAGCATGAATAGTAGCAATTACTGCTGTGAATTGTGTTGTA 715
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
estA3:   181 GTAATAAAAGTGGTCCTGAAAGCATGAATAGTAGCAATTACTGCTGTGAATTGTGTTGTA 240
pPM4565  716 ATCCTGCTTGTACCGGGTGCTATTAATAATATAAAGGGAACTAAACAGTTCCCTTTATAT 775
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
estA3:   241 ATCCTGCTTGTACCGGGTGCTATTAATAATATAAAGGGAACTAAACAGTTCCCTTTATAT 300
pPM4565  776 TTGTTCTGATTCTGATGATGTCTGTAACGTATGTACCTGTTGCTTTGTTGAATAAATCGA 835
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
estA3:   301 TTGTTCTGATTCTGATGATGTCTGTAACGTATGTACCTGTTGCTTTGTTGAATAAATCGA 360
```

Transposon Tn*602* IS602L region DNA, 5' end.

```
pPM4565  817 GCTTTGTTGAATAAATCGAACTTTCGCTGGGTTGAAGGAGCAGAGCACGCATCATCCGG 875
             |||||||||||||||||||||||| |||| ||||||||| |||| |||| ||| ||| |
Tn602:    12 GCTTTGTTGAATAAATCGAACTTTTGCTGAGTTGAAGGATCAGATCACGTATCTTCCCG 70
pPM4565  872 CCGGCAACATGAGTCGTTCCATGGTAAAGCAGAAGTTCAGAATCACCAACTGGCGC 927
             ||| ||||      |||||| ||| |||||| ||||||| |||||||||||||| |
Tn602:    68 CCGACAACGCAGACCGTTCCGTGGCAAAGCAAAAGTTCAAAATCACCAACTGGCCC 123
```

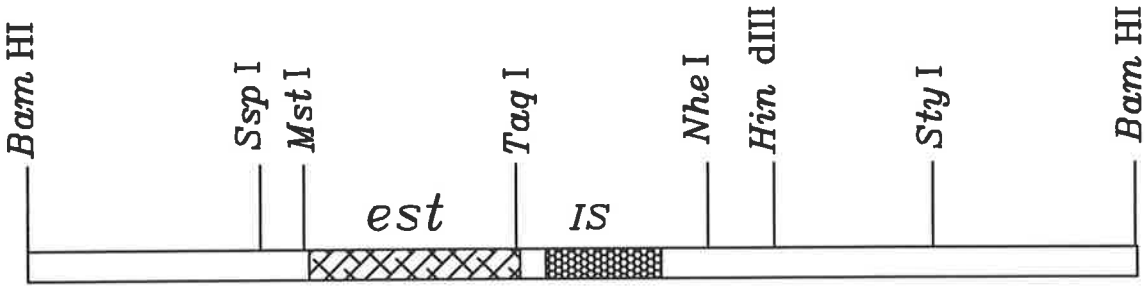
***E. coli* transposable element variant IS*903*.B present on kanamycin resistance transposon Tn 2680**
Identities = 177/218 (81%), Strand = Plus / Plus

```
pPM4565  872 CCGGCAACATGAGTCGTTCCATGGTAAAGCAGAAGTTCAGAATCACCAACTGGCGCAGCC 931
             ||| ||||      |||||| ||| |||||| ||||||| |||||||||||||| || |
IS903:   179 CCGACAACGCAGACCGTTCCGTGGCAAAGCAAAAGTTCAAAATCACCAACTGGCCCACCT 238
pPM4565  932 ACAACAAAGCCCTTATCCTCCGTGGCACCATCACTTTCTGGCGGGATGGCGAGGCAATCC 991
             |||| |||||||| |||  ||||||| |||| |||||||||| |||||  || || || |
IS903:   239 ACAATAAAGCCCTCATCAACCGTGGCTCCATAACTTTCTGGCTGGATGATGAAGCTATTC 298
pPM4565  992 AGGCCTGGTATGAGTCAGCAACCCCCTCATCACGGGGACGACCTCAGAGTTATTCTGACC 1051
             |||||||||||||||||||||| || || ||||| ||  |||||||| | ||||||||||
IS903:   299 AGGCCTGGTATGAGTCAGCAACACCTTCTTCACGAGGCAGACCTCAGCGCTATTCTGACC 358
pPM4565 1052 TTGCTATTACCACTGCCCTTGTGATTAAACGCGTATTC 1089
             |||| || || ||||  || || ||||||||||||||
IS903:   359 TTGCCATCACGACTGTGCTGGTCATTAAACGCGTATTC 396
```

Transposon Tn*903* Identities = 176/218 (80%), Strand = Plus / Plus

```
pPM4565  872 CCGGCAACATGAGTCGTTCCATGGTAAAGCAGAAGTTCAGAATCACCAACTGGCGCAGCC 931
             ||| ||||      |||||| ||| |||||| ||||||| |||||||||||||  || |
Tn903:  2095 CCGACAACGCAGACCGTTCCGTGGCAAAGCAAAAGTTCAAAATCACCAACTGGTCCACCT 2154
pPM4565  932 ACAACAAAGCCCTTATCCTCCGTGGCACCATCACTTTCTGGCGGGATGGCGAGGCAATCC 991
             |||||||||| || ||| |||||||  || |||||||||||| |||||  | ||| || |
Tn903    2155 ACAACAAAGCTCTCATCAACCGTGGCTCCCTCACTTTCTGGCTGGATGATGGGGCGATTC 2214
pPM4565  992 AGGCCTGGTATGAGTCAGCAACCCCCTCATCACGGGGACGACCTCAGAGTTATTCTGACC 1051
             |||||||||||||||||||| || || ||||| ||  |||||||| | ||||||||||
Tn903:  2215 AGGCCTGGTATGAGTCAGCAACACCTTCTTCACGAGGCAGACCTCAGCGCTATTCTGACC 2274
pPM4565 1052 TTGCTATTACCACTGCCCTTGTGATTAAACGCGTATTC 1089
             |||| || || ||||  || || ||||||||||||||
Tn903:  2275 TTGCCATCACGACTGTGCTGGTCATTAAACGCGTATTC 2312
```

C



7.2.1.3 Features of plasmid pPM4565

The plasmid pPM4565 which encodes ST contains a fragment of 1.79 kb. The homology of this fragment with published sequences was investigated using the BLASTN program (Fig 7.4C). A region of 297 bp (nt 448-744) has 65% homology with transposon Tn*1681* encoding STA1 (So and McCarthy, 1980). The region including nucleotides 476-835 is identical to *E. coli estA3* gene (Moseley *et al.*, 1983). The region downstream of *est* showed high homology with several insertion sequence elements: nucleotides 817-927 have 83% homology with the 5' end of Tn*602*: nt 872-1089 are homologous (81%) to the 5' end (nt 179-396) of the *E. coli* transposable element IS903.B present on the kanamycin resistance transposon Tn*2680* and to the 3' end (2095-2312) of Tn*903* (Fig7.4C).

7.2.1.4 Construction of an *est* gene cassette

Since it was planned to introduce a fragment of the *est* gene encoding the mature ST into the *cstH* gene, it was necessary to remove the stop codon and introduce desired restriction sites at the ends of the *est* gene so that it could be inserted in-frame into *cstH*. Oligos # 738 and 739 were designed to allow PCR amplification of the mature toxin coding region of the gene in such a way that they also changed the stop codon and introduced *Xba*I sites that would allow correct insertion of the *est* gene fragment into the mutant *cstH*. Polymerase chain reaction (PCR) was performed using these oligos as primers to amplify a fragment of 88 bp. The PCR product was purified, and digested with *Xba*I, and cloned into the *Xba*I site of pBluescript SK. Transformants were screened for the *est* gene sequence using an *est*-specific Dig-labelled oligo #814. Plasmid DNAs from some positive clones were digested with *Pvu*II and *Xba*I to check the presence of the correct fragment. One positive clone was further characterized by

DNA sequencing. The plasmid in this clone containing DNA encoding the 19 aa mature ST was designated pPM4566 and used as a source of the *est* cassette for insertion into mutant *cstH* genes (Fig. 7.5).

7.2.2 Insertion of *est* into *cstH*

In order to insert *est* into *cstH*, pPM4566 DNA or the PCR product from oligos #738 and #739 was digested with *Xba*I, the *est* fragment purified and cloned into either the *Xba*I or *Sty*I sites (between nt 4413-4414 and 4515-4516, respectively) of the mutant *cstH* genes, (plasmids pPM4556 and pPM4557). Insertion into a third site (between nt 4542-4543 of *cstH'* in plasmid pPM4559) was not successful suggesting that this is not a permissive site. Transformants were screened using Dig-labelled oligo # 814, and the in-frame insertion and orientation of *est* in *cstH* were confirmed by sequencing of the fusions. Two plasmids designated pPM4569 and pPM4574 (Fig. 7.6) with in-frame insertions were selected, and used for further studies. Computer analysis of the secondary structure and solvent accessibility of the hybrid proteins using the PHD program suggested that the foreign epitope would be present as a loop in the hybrid proteins and exposed to the environment (Fig. 7.7). The data suggest that the insert would be expressed on the surface of the protein and eventually on the surface of both the hybrid pili and the bacteria.

7.2.3 Characterization of CstH::ST hybrid proteins

7.2.3.1 Expression of CstH::ST in DH5α

E coli K-12 strain DH5α harbouring plasmids pPM4569 and pPM4574 encoding CstH::ST hybrid proteins were analysed for expression by Western blot

Fig. 7 5 Construction of plasmid pPM4566.

An 85 bp fragment encoding mature ST was amplified from pPM4565 by PCR using oligos #738 and #739 into which *Xba*I sites had been incorporated. The PCR product was digested with *Xba*I and ligated into the *Xba*I site of pBluescript SK. Transformants were screened by DNA hybridization using Dig-labelled oligo #814 as a probe and confirmed by sequencing. Plasmid pPM4566 harbours the *est* gene cassette.

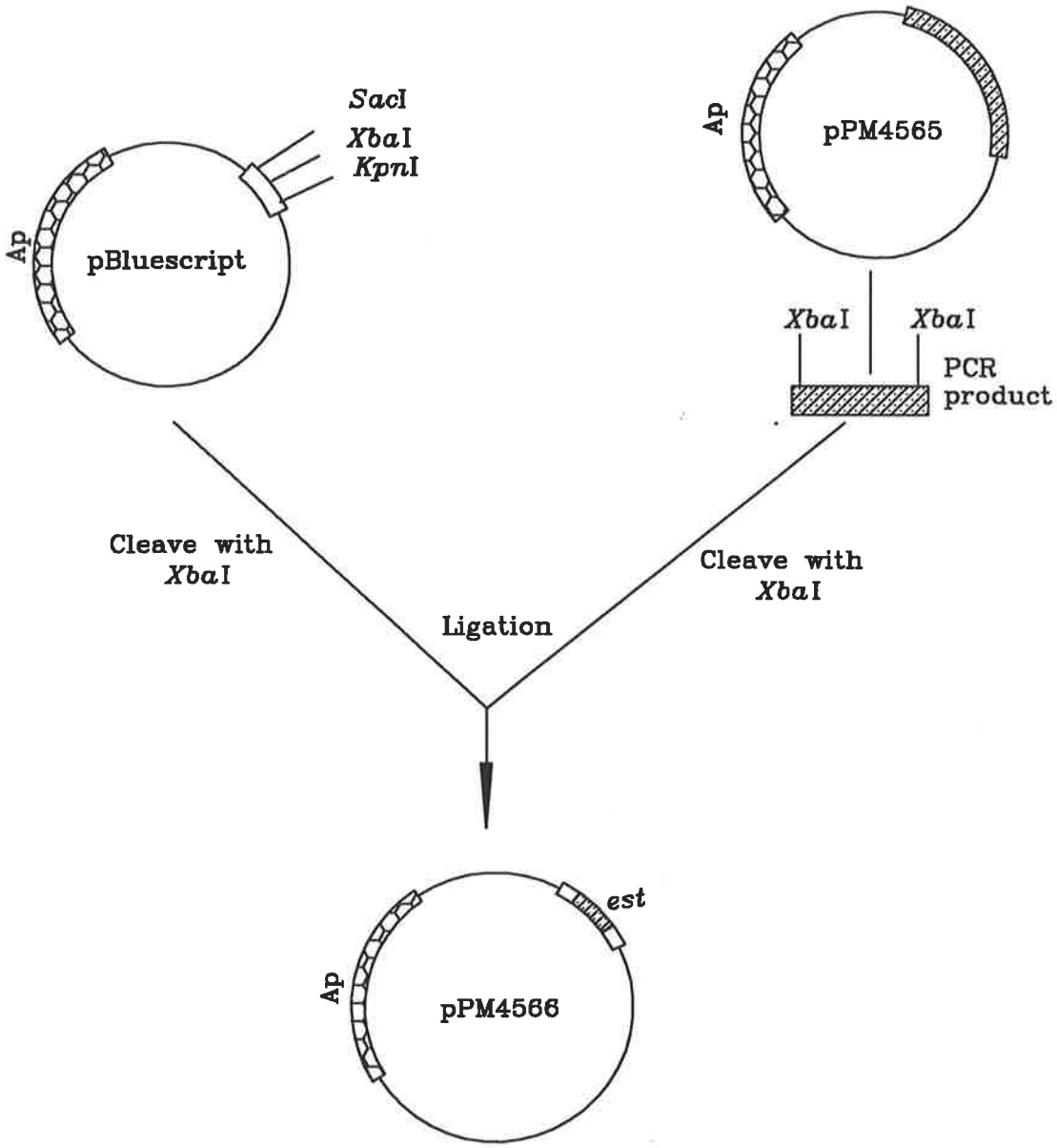

SacI
XbaI
KpnI
Ap
pBluescript
Ap
pPM4565
XbaI
XbaI
PCR
product
Cleave with
XbaI
Cleave with
XbaI
Ligation
est
Ap
pPM4566

Fig. 7.6 Insertion of the *est* cassette into *cstH*.

The *est* cassette was inserted into the mutant *cstH* genes either using PCR product (A) or by cleavage of the fragment from pPM4566 (B). Transformants were screened by hybridization to Dig-labelled oligo #814 and sequencing. Plasmids produced were called pPM4569 and pPM4574.

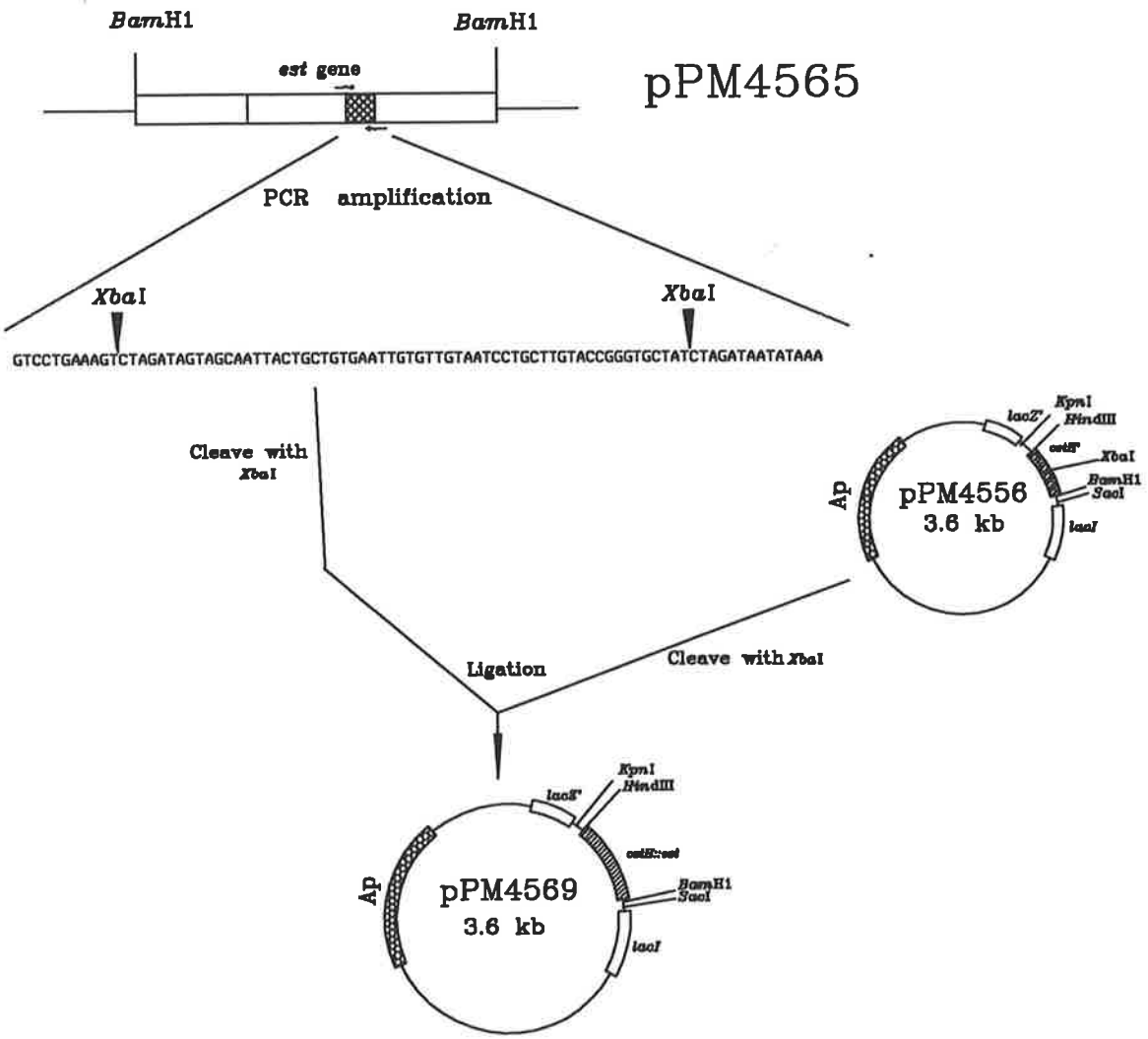
A:

A fragment of 88 bp encoding mature ST was amplified by PCR using oligos #738 and #739 and cleaved with *Xba*I. This fragment was then ligated into the XbaI cleaved pPM4556. The resultant plasmid was designated pPM4569.

B:

The *est* cassette was liberated from pPM4566 by cleavage with *Xba*I. This was then ligated to pPM4557 cleaved with *Sty*I. The resultant plasmid was designated pPM4574.

A


BamH1
BamH1
est gene
pPM4565
PCR amplification
XbaI
XbaI
GTCCTGAAAGTCTAGATAGTAGCAATTACTGCTGTGAATTGTGTTGTAATCCTGCTTGTACCGGGTGCTATCTAGATAATATAAA
Cleave with XbaI
pPM4556
3.6 kb
Ap
lacZ'
KpnI
HindIII
estE'
XbaI
BamH1
SacI
lacI
Ligation
Cleave with XbaI
pPM4569
3.6 kb
Ap
lacZ'
KpnI
HindIII
estE::est
BamH1
SacI
lacI

B

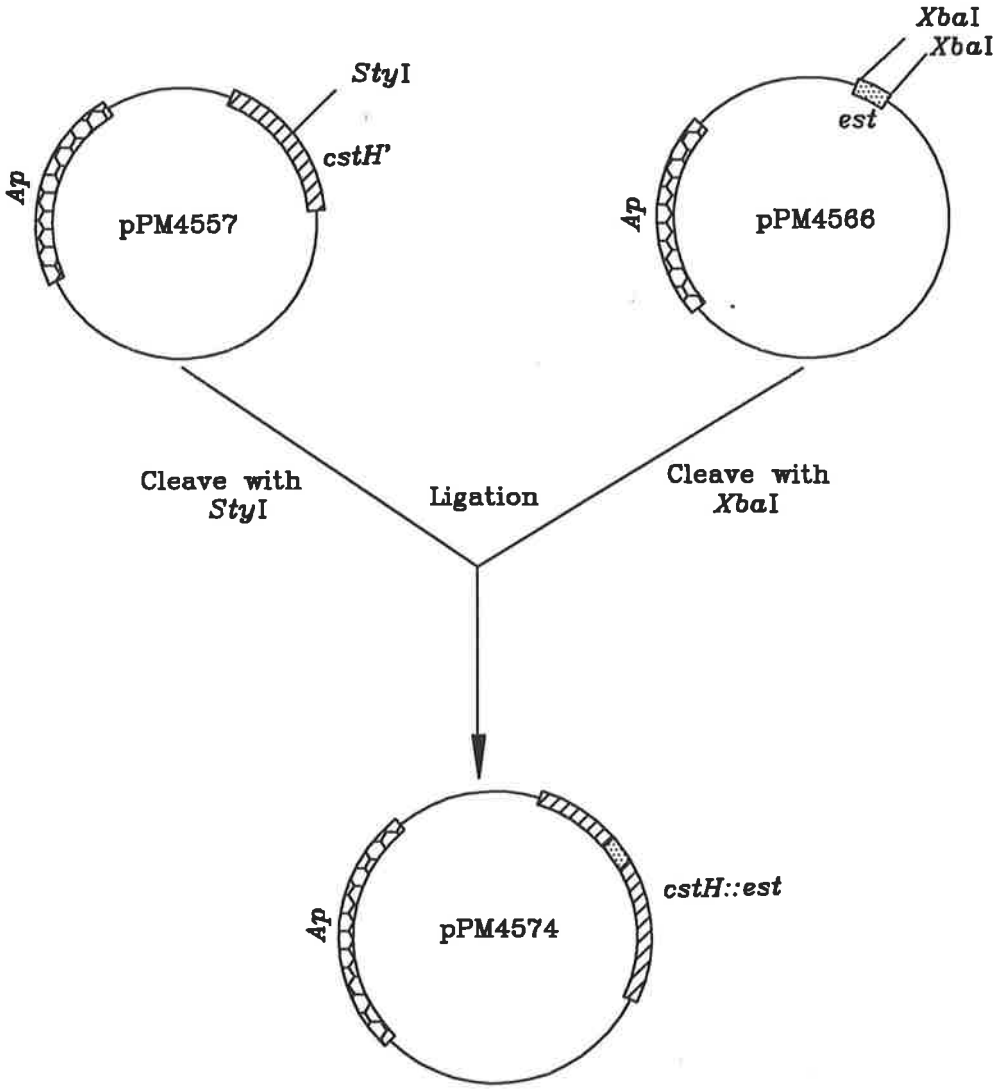

StyI
cstH'
Ap
pPM4557
XbaI
XbaI
est
Ap
pPM4566
Cleave with StyI
Ligation
Cleave with XbaI
cstH::est
Ap
pPM4574

Fig. 7.7 Secondary structure analysis of the amino acid sequence of the CstH::ST hybrid proteins

The secondary structure and solvent accessibility of the CstH::ST hybrid proteins was determined using the PHD program (Rost and Sander, 1993; 1994). Panel A indicates the hybrid protein with insert between amino acids 65-66 encoded by plasmid pPM4569, and panel B the hybrid protein with an insert between amino acids 99-100 encoded by pPM4574. The underlined, italic sequences correspond to the inserts.

The abbreviations are the same as in Fig.6.4.

A

prediction of: secondary structure-solvent accessibility of the Cst::ST fusion in pPM4569

```
                 ....,....10...,....20...,....30...,....40...,....50...,....60
        AA       |AAGPTLTKELALNVLSPAALDATWAPQDNLTLSNTGVSNTLVGVLTLSNTSIDTVSIAST|
        PHD sec  |      E EEHHHE                          EEEEEEEEEEE     EEEE   |
        Rel sec  |99922101111111137744037775788511689712489999999725774476378 9|
        prH sec  |00034444444454210123431112100000000000000000000000000000000|
        prE sec  |00012344444444321000000000012441001456889999987421126876100|
        prL sec  |99842100001000357866468886888755897543100000001578762113888|
subset: SUB sec  |LLL.............LL....LLLLLLLL..LLLL...EEEEEEEEE.LLL..EE.LLL|

ACCESSIBILITY
3st:    P_3 acc  |eeeeebbeebbbbbbbbbebeeeeeeeeeeeebeeeebbebbbbbbbbeeee ebbeebbe|
10st:   PHD acc  |99777007600000000607889777779707979007000000006777570077007|
        Rel acc  |95277515265719762012554354334280485510325935924134404015501 7|
subset: SUB acc  |ee.eeb.e.bbb.bbb....eee.ee..e.e.eeee....bb.bb.b..ee.e..ee..e|

                 ....,....70...,....80...,....90...,....100..,....110..,....120
        AA       |NVSDTLDSSNYCCELCCNPACTGCYLENGTVTFAHETNNSASFATTISTDNANITLDKNA|
        PHD sec  |       EEE          EE   EEEEEEE     EEEEEEE    EEEEE    |
        Rel sec  |88886899986471112875221112166699974157985045998356635765436 8|
        prH sec  |00000000000000010112433321100000000000001000000000000000000|
        prE sec  |00001000001674443000001234322799986521002466886211367766310|

        prL sec  |88887899987314545877554333477100012478886422001367762122257 8|

subset: SUB sec  |LLLLLLLLLLL.E....LLL.......LLEEEEE..LLLLL..EEEE.LLL.EEEE..LL|

ACCESSIBILITY
3st:    P_3 acc  |ebbeebeeeebbbbbbb bbbbbbbbeebebbbb e eeebbbbbbbbbe bebbbeeeb|
10st:   PHD acc  |70077077770000000500000000790600005757670000000006506000677 0|
        Rel acc  |60143163440991898157755811752143751403134147233101051405144 2|
subset: SUB acc  |e..e..e.ee.bb.bbb.bbbbbb..ee..b.bb.e....b.bb.......b.b.b.ee.|

                 ....,....130..,....140..,....150..,....160..,166
        AA       |GNTIVKTTNGSQLPTNLPLKFITTEGNEHLVSGNYRANITITSTIK|
        PHD sec  |  EEEEE           EEEEE   EEEEEE E EEEEEEEEEE |
        Rel sec  |97799933897689887717887457278981111699988757 49|
        prH sec  |00000000000000000000000000000000000000000000000|
        prE sec  |01899963101110001157887321588884444788888877 60|
        prL sec  |88100036898788888841001677411014545100010121 39|
subset: SUB sec  |LLEEEE..LLLLLLLLLL.EEEE.LL.EEEE....EEEEEEEEE.L|

ACCESSIBILITY
3st:    P_3 acc  |eebbbebbe eebbeebbbebbbeeb ebbbbbeb bbbbbbbebe|
10st:   PHD acc  |96000602757600960006000670570000062500000006 09|
        Rel acc  |42175220403220410033352151150661000162608032 16|
subset: SUB acc  |e..bb...e.....e......b..e..e.bb.....b.b.b....e|
```

B

prediction of: secondary structure-solvent accessibility of the Cst::ST fusion in pPM4574

```
.....,...                  .10...,....20...,....30...,....40...,....50...,....60
         AA       |AAGPTLTKELALNVLSPAALDATWAPQDNLTLSNTGVSNTLVGVLTLSNTSIDTVSIAST|
         PHD sec  |      HHHHHHHH   HHH          EEEE   EEEEEEEEEEEE       E   |
         Rel sec  |99984214776422421132124455663267244548657899999727789853235 7|
         prH sec  |00002345777654234455332222111100000000111000000000000001100|
         prE sec  |00001122001011011111111111002567523268768889999851100023422 1|
         prL sec  |99985432111233654433445566775321366631110000000148888875357 8|
subset:  SUB sec  |LLLL....HHH.............LLLL..EE...L.EEEEEEEEEE.LLLLLL...LL|

ACCESSIBILITY
3st:     P_3 acc  |eeeeebbeebbbebbbbbbbebbbbeeeebebbeebbbbbbbbbbbbbe eeeeeeeebe|
10st:    PHD acc  |99777007600060000000700006777060076000000000000756777776707|
         Rel acc  |94355315247415641353550031443224021053368979948340135344042 3|
subset:  SUB acc  |ee.ee..e.bbb.bbb..b.eb....ee...b....b..bbbbbbbb.e...e.ee.e..|

                   ....,....70...,....80...,....90...,....100..,....110..,....120
         AA       |NVSDTSKNGTVTFAHETNNSASFATTISTDNANITLDKNLDSSNYCCELCCNPACTGCYL|
         PHD sec  |          EEEEEEE                                EEEE E      |
         Rel sec  |54353668724888514989999964145775535566789997178312386422123 3|
         prH sec  |00000000000000000000000000111112232211000000000000022333222|
         prE sec  |22323110156888743010000012421100000001000015786453000001322|
         prL sec  |66666778732101246889899876466776656777889988411354587655444 5|
subset:  SUB sec  |L..L.LLLL..EEEE..LLLLLLLL...LLLLL.LLLLLLLLLLL.EE....LL......|

ACCESSIBILITY
3st:     P_3 acc  |eebeebeeeebebbeebeeeee eeebbee beeebeeebeeeebbbbbbbbbbbbbbbb|
10st:    PHD acc  |76076077660607709777757760066507760787287770000000000000000|
         Rel acc  |71031275111153550456530331421100431246406333099289906887880 2|
subset:  SUB acc  |e.....ee....b.ee.eeee.....b.....e...eee.e....bb.bbb.bbbbbb.|

                   ....,....130..,....140..,....150..,....160..,166
         AA       |GNTIVKTTNGSQLPTNLPLKFITTEGNEHLVSGNYRANITITSTIK|
         PHD sec  |  EEEEE           EEEEE   EEEEE  E EEEEEEEEEE |
         Rel sec  |42899923898579887717886456178981111698988757 49|
         prH sec  |10000000000000000000000000000000000000000000000|
         prE sec  |23899853100210001157887322578884444788888776 0|
         prL sec  |65100045898788888841001677410014545200011121 39|
subset:  SUB sec  |..EEEE..LLLLLLLLLL.EEEE.LL.EEEE....EEEEEEEEE.L|

ACCESSIBILITY
3st:     P_3 acc  |b bbbebbe eebbeebbbebbbeeb ebbbbbeb bbbbbbbebe|
10st:    PHD acc  |05000602757600960006000670570000062500000006 09|
         Rel acc  |30055220403220410033352151150661000162608032 16|
subset:  SUB acc  |...bb...e.....e......b..e..e.bb.....b.b.b....e|
```

analysis using antisera against native CS3 and a MAb against mature ST. In no cases could the hybrid proteins be detected (Fig. 7.8 trackes 1-4).

Since the hybrid proteins were not expressed in *E. coli* K-12 strain DH5α, the same reasons as for the failure of expression of the CstH::LT-B_{44-64} (Chapter 6) were considered and it was decided to attempt to overcome them by expression of the hybrid proteins in different systems.

7.2.3.2 Expression of constructs in a *degP* strain

It was expected that the hybrid proteins would be located in the periplasm or on the periplasmic side of the cytoplasmic membrane. Hence, in order to protect the proteins from degradation, plasmids encoding the hybrid proteins were transformed into an *E. coli* K-12 strain, Ecc219 (Table 2.2), which is deficient in periplasmic protease Deg^P. Expression of the hybrid proteins was analysed by Western blot using a polyclonal antibody against CS3 and MAb against ST. None of the proteins could be detected (Fig. 7.8 tracks 9-10).

7.2.3.3 Expression of constructs together with the assembly genes

PB176p^-, a mutant from ETEC strain PB176 deficient in the expression of CFA/II , ST and LT (Evans, 1979), was used to express the hybrid proteins. The plasmids encoding the hybrid proteins, and the CS3 assembly cassette (section 6.2.1.2) were electroporated into PB176p^- and *E. coli* K-12 strain DH5α. Transformants were studied for the expression of the hybrid proteins by Western blot analysis using a polyclonal antiserum to CS3 and Mabs against CstH and ST. In no case could the hybrid proteins be detected (Fig. 7.8, tracks 5-8). In all of the above experiments the

Fig. 7.8 Immunoblot analysis of hybrid CstH proteins.

The CstH::ST hybrid proteins were expressed in different strains and analysed by Western blot. Whole cell lysates were electrophoresed in SDS on 15% polyacrylamide gels, transferred to a nitrocellulose filter and probed with an antiserum against CS3. The blots were visualized using ECL. The arrow corresponds to the position of CstH.

Lanes:

1. PB176 (+control)
2. *E. coli* K-12[pBluescript] vector plasmid for *cstH::est* (-control)
3. *E. coli* K-12[pPM4569] encoding CstH::ST (between aa 65-66)
4. *E. coli* K-12[pPM4574] encoding CstH::ST (between aa 99-100)
5. *E. coli* K-12[pPM4569] encoding CstH::ST (between aa 65-66) and CS3 assembly genes
6. *E. coli* K-12[pPM4574] encoding CstH::ST (between aa 99-100) and CS3 assembly genes
7. PB176p$^-$[pPM4569] encoding CstH::ST (between aa 65-66) and CS3 assembly genes
8. PB176p$^-$[pPM4574] encoding CstH::ST (between aa 99-100) and CS3 assembly genes
9. *E. coli* K-12 Ecc219[pPM4569] encoding CstH::ST (between aa 65-66)
10. . *E. coli* K-12 Ecc219[pPM4574] encoding CstH::ST (between aa 99-100)


1 2 3 4 5 6 7 8 9 10

immunoblots were detected by Enhanced Chemiluminescence (ECL) (Boehringer Mannheim) which is particularly sensitive for the detection of small amounts of protein.

These results indicate that the hybrid proteins are not expressed in this system at a level that could be detected with antibody. In addition analysis in a *deg*^{P} background suggested that this is not because of degradation of the proteins by this protease. It was thus decided to use an alternative expression system based on the T7 promoter/RNA polymerase system (Tabor and Richardson, 1985) for expression of the hybrid proteins.

7.2.3.4 Expression of the constructs in the T7 promoter /RNA polymerase system

In order to overcome the potential problems related to the transcription and translation of the hybrid genes, an expression system based on the T7 promoter/RNA polymerase system (Tabor and Richardson, 1985) was used. The DNA fragment in pPM4574, was re-cloned into the plasmid pBluescript KS to give plasmid pPM4576 (Fig. 7.9) in order to place the *cstH* hybrid gene under control of the T7 promoter.

For analysis of the expression of the hybrid proteins, pPM4576 and pGP1-2 were transformed into *E. coli* K-12 strain MZ1 (Zuber *et al.,* 1987) selecting for Ap^{R} and Km^{R} and the presence of the two plasmids was confirmed by restriction analysis. The expression of the proteins was studied by Western blot analysis using a polyclonal antiserum to CS3 and Mabs to CstH and to ST. Two hybrid proteins (ST inserted between residues 65-66 and 99-100 in mature CstH) reacted with antibodies to both CS3 and ST (Fig. 7.10 and 7.11). Unexpectedly the two hybrid proteins had different mobilities on PAGE, which may be due to the differences in their amino acid sequences. This result shows that CstH can tolerate the insertion of a complex foreign polypeptide in two sites and that insertion in these regions does not dramatically change the overall

Fig. 7 9 Cloning of *cstH::est* under the control of the T7 promoter.

Plasmid pPM4574 was digested with *Bam*HI and *Hind*III and the DNA fragment containing *cstH::est* and the *cstH* promoter was ligated between the *Bam*HI and *Hin*dIII sites of pBluescript KS. The resultant plasmid was designated pPM4576 and contains *cstH::est* under the control of the T7 promoter.

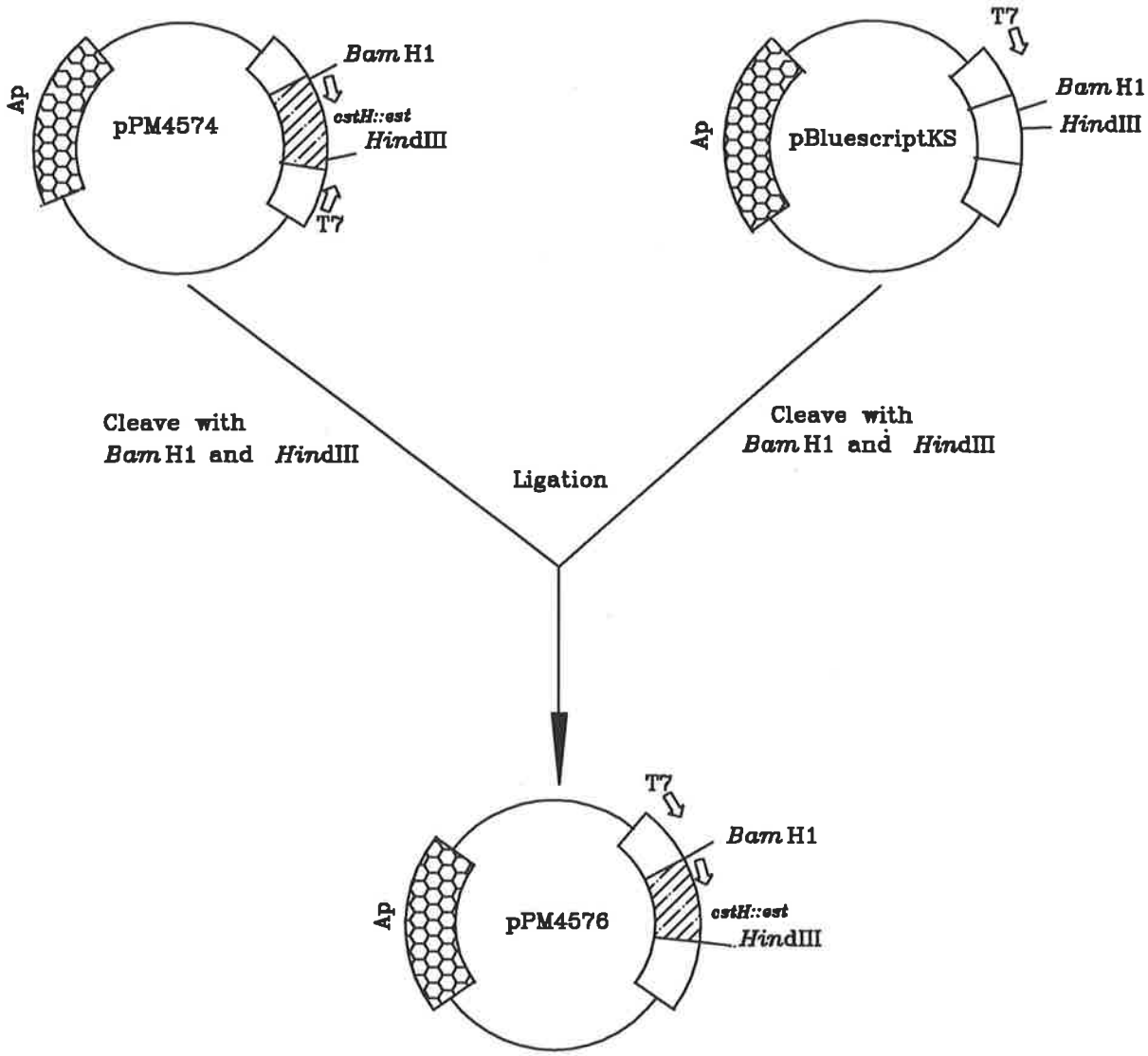

Ap
pPM4574
Bam H1
cstH::est
HindIII
T7
T7
Bam H1
HindIII
Ap
pBluescriptKS
Cleave with
Bam H1 and HindIII
Ligation
Cleave with
Bam H1 and HindIII
T7
Bam H1
Ap
pPM4576
cstH::est
HindIII

Fig. 7.10 Immunoblot analysis of hybrid CstH proteins.

The CstH::ST hybrid proteins were expressed in the T7 promoter/RNA polymerase system and analysed in a Western blot. Whole cell lysates were run in SDS on 15% polyacrylamide gels, transferred to a nitrocellulose filter, probed with a polyclonal antiserum against CS3 (Panel A) and Mab CH1 to CstH (Panel B) and the blot visualized by ECL. The left arrow corresponds to CstH.

Lanes:

A:

1. PB176 (+control)

2. PB176p$^-$ (-control)

3. *E. coli* K-12[pPM4556] expressing mutant CstH with mutation in nt#4413-4417 *cstH*

4. *E. coli* K-12[pPM4569] expressing CstH::ST (between aa 65-66)

5. *E. coli* K-12[pPM4576] expressing CstH::ST (between aa 99-100)

B:

1. PB176 (+control)

2. PB176p$^-$ (-control)

3. *E. coli* K-12[pPM4569] expressing CstH::ST (between aa 65-66)

4. *E. coli* K-12[pPM4576] expressing CstH::ST (between aa 99-100)

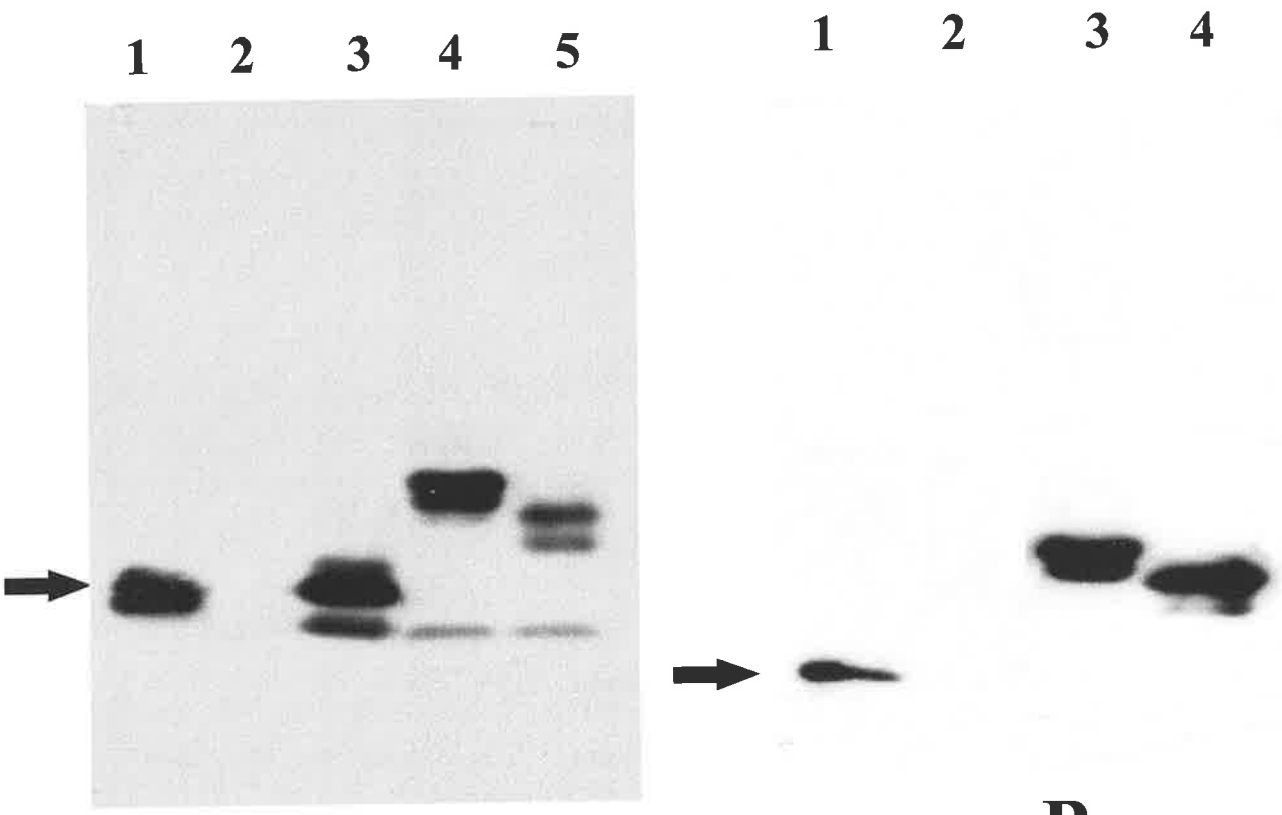

1 2 3 4 5
1 2 3 4
A
B

Fig. 7.11 Immunoblot analysis of the hybrid CstH proteins.

The CstH::ST hybrid proteins were expressed in the T7 promoter/RNA polymerase system and analysed in a Western blot. Whole cell lysates were run in SDS on 15% polyacrylamide gels, transferred to a nitrocellulose filter, probed with Mab 1:3 to ST and the blot visualized by ECL. The arrow corresponds to the position of the CstH:ST hybrid proteins.

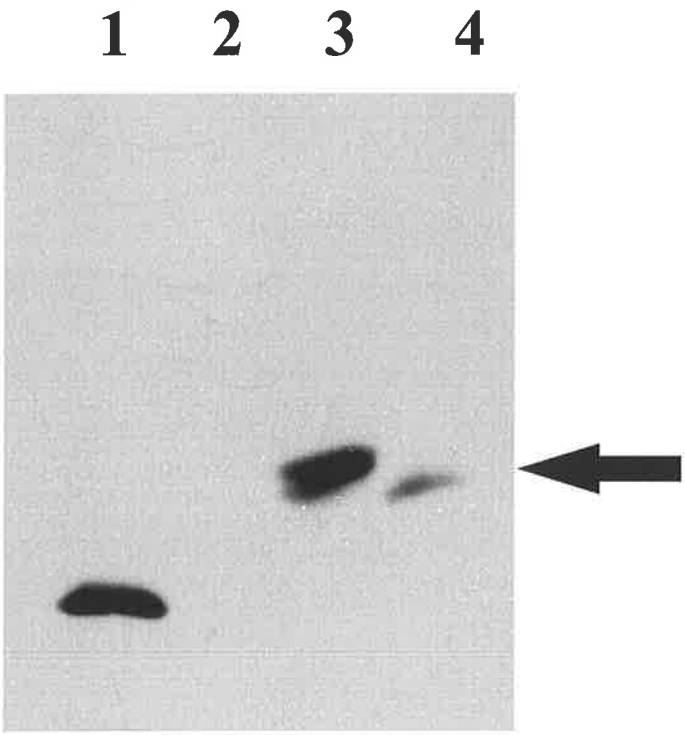
Lanes:

1. PB176 (+control)

2. PB176p$^-$ (-control)

3. *E. coli* K-12[pPM4569] expressing CstH::ST (between aa 65-66)

4. *E. coli* K-12[pPM4576] expressing CstH::ST (between aa 99-100)


1
2
3
4

antigenic structure of CstH. In addition it also demonstrated that foreign epitopes can be inserted in these regions and be expressed as hybrid proteins.

7.2.4.1 Cloning of the hybrid *cstH* gene into the *cst* operon.

In the above sections it has been shown that the expression of the hybrid proteins from a T7 promoter provided sufficient protein to be detected by Western blot. It was thought that the native biosynthetic operon structure of CS3 may enhance the poor expression of the hybrid proteins. Therefore the hybrid *cstH::est* genes were used to replaced the wild type *cstH* gene in the *cst* operon.

Plasmids pPM4569, pPM4574 (encoding CstH::ST hybrid proteins) and pPM484 were digested with *Bsu*36I and *Bam*HI and the 4.3 kb fragment containing the CS3 assembly genes was cloned between the *Bsu*36I and *Bam*H1 sites of plasmids pPM4569 and pPM4574 to give plasmids pPM4571 and pPM4577, respectively (Fig. 7.12). In the plasmid pPM4571 the recombinant *cst* operon is orientated so that it can be expressed from the T7 promoter/RNA polymerase system if necessary.

7.2.4.2 Expression of the CstH::ST hybrid proteins from the CS3 operon

Expression of the hybrid proteins was investigated by Western blot using a polyclonal antiserum to CS3 and MAb against CstH. Bacteria harbouring plasmids pPM4571 and pPM4577 encoding the hybrid CstH::ST with ST inserted between amino acids 65-66 and 99-100, respectively, were grown overnight on CFA containing Ap. Using cell lysates from $5x10^9$ cells in Western blot analysis with both Mab and polyclonal antiserum, as shown in fig 7.13, it was possible to detect bands corresponding to the hybrid proteins (Fig. 7.13).

Fig. 7.12 Construction of hybrid *cst* operons

Plasmids pPM484, pPM4569 and pPM4574 were digested with *Bam*HI and *Bsu*36I and the DNA fragment containing the CS3 assembly genes from pPM484 was cloned between the *Bam*HI and *Bsu*36I sites of pPM4569 and pPM4574 upstream of *cstH:est* to generate pPM4571 and pPM4577, respectively.

Abbreviation:

r Recombinant

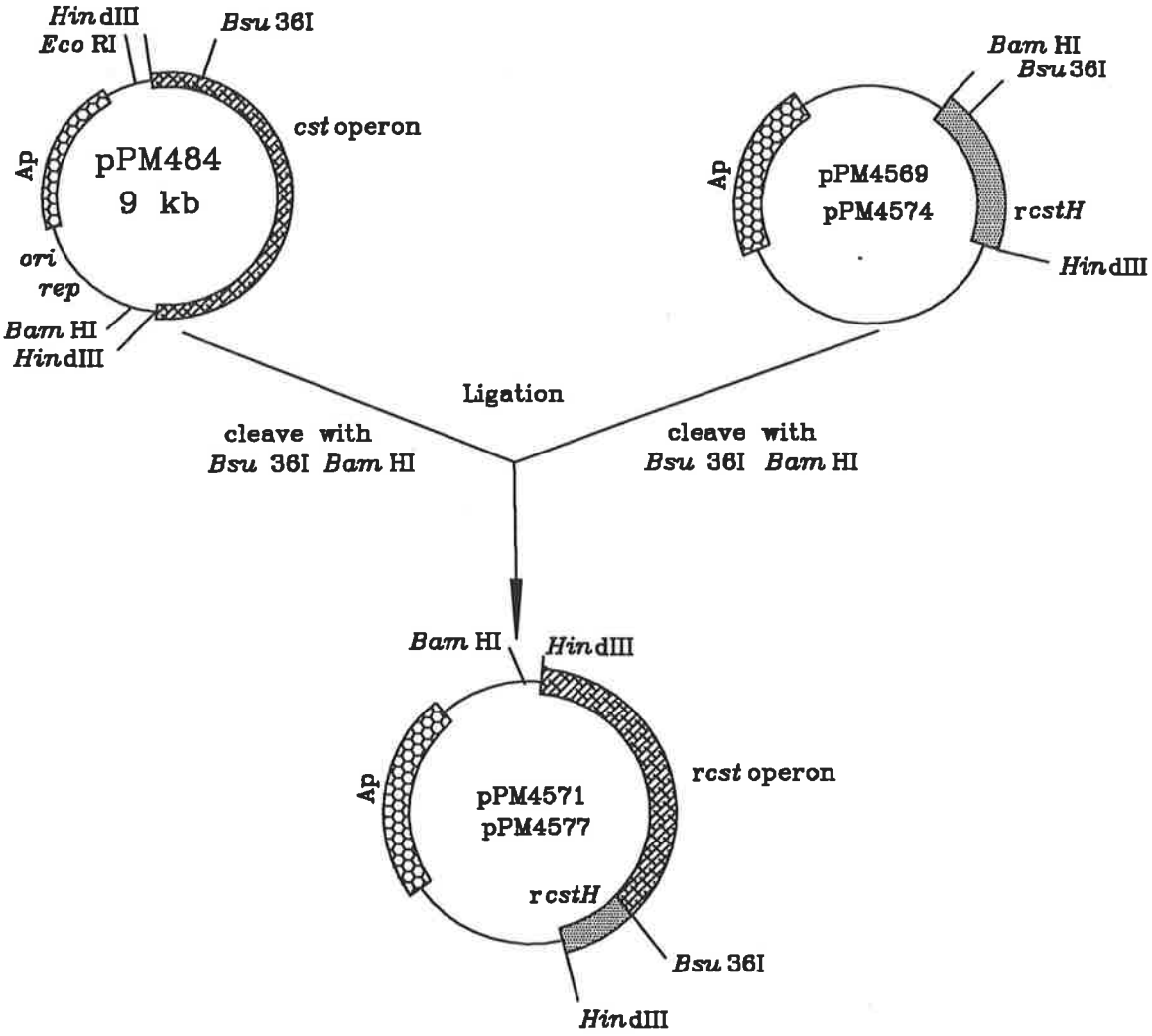
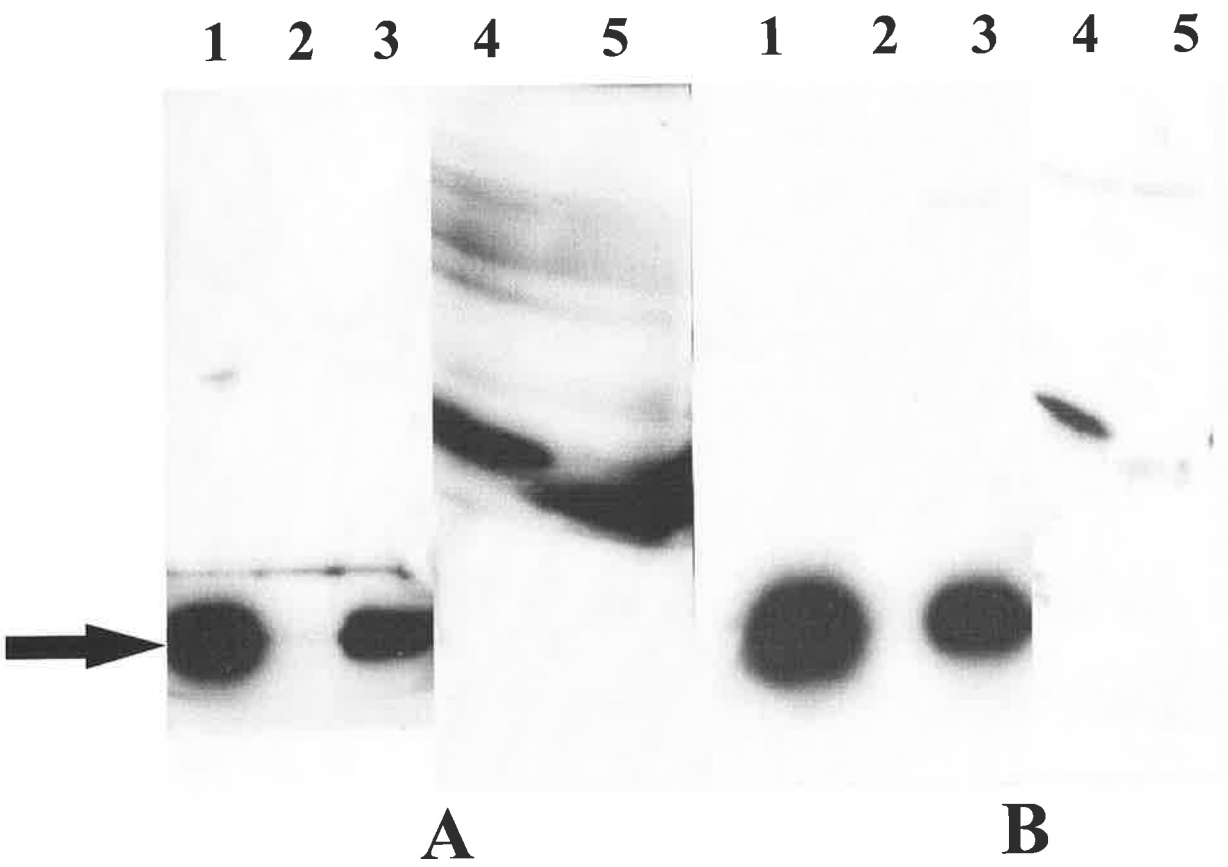

HindIII
Eco RI
Bsu 36I
cst operon
Ap
pPM484
9 kb
ori
rep
Bam HI
HindIII
Bam HI
Bsu 36I
Ap
pPM4569
pPM4574
rcstH
HindIII
Ligation
cleave with
Bsu 36I Bam HI
cleave with
Bsu 36I Bam HI
Bam HI
HindIII
Ap
pPM4571
pPM4577
rcst operon
rcstH
Bsu 36I
HindIII

Fig. 7.13 Immunoblot analysis of hybrid CstH proteins expressed from the recombinant *cst* operon.

The CstH::ST hybrid proteins were expressed from the recombinant *cst* operon and analysed by Western blot. Whole cell lysates were electrophoresed in SDS on 15% polyacrylamide gels, transferred to a nitrocellulose filter and probed with polyclonal antiserum to CS3 (Panel A) and Mab CH10 to CstH (Panel B). The arrow corresponds to the position of CstH.

Lanes:

1. PB176 (+control)
2. *E. coli* K-12[pBluescript] vector plasmid for *cstH::est* (-control)
3. *E. coli* K-12[pPM484] expressing wild type CstH
4. *E. coli* K-12[pPM4571] expressing CstH::ST (between aa 65-66)
5. *E. coli* K-12[pPM4577] expressing CstH::ST (between aa 99-100)


1 2 3 4 5
1 2 3 4 5
A
B

7.2.5 Re-cloning and expression of the mutant *cst* operon in plasmid pBC KS

As indicated in the previous chapter (section 6.2.7) the vector pBC KS improved the expression of CstH::LT-B_{44-64} hybrid proteins, consequently it was decided to check the expression of the CstH::ST hybrid proteins in this system.

The mutant *cst* operons were cloned into plasmid pBC KS. Purified *Hin*dIII fragments encoding mutant *cst* operons from plasmids pPM4571 and pPM4577 were ligated into the *Hin*dIII site of pBC KS to give plasmids pPM4573 and pPM4579, respectively (Fig. 7.14). The ligations were transformed into *E. coli* K-12 strain DH5α and transformants were screened for Cm^R and by restriction digestion analysis. Clones resistant to Cm and containing plasmids of the correct size were selected for further investigation. The orientation of the cloned DNA in the plasmid vector was confirmed by restriction enzyme digestion of the constructs. Plasmids with the recombinant *cst* operon in the T3 orientation were used for expression of the hybrid CstH proteins.

Western blot analysis of the expression from the recombinant *cst* operon using a polyclonal antiserum to CS3 and MAb against CstH revealed bands corresponding to the CstH hybrid proteins (Fig. 7.15) and with higher molecular weight than CstH.

Comparison of the level of the CstH hybrid proteins expressed from pBluescript and pBC KS indicated that the pBC KS vector led to greater production of proteins, particularly for the hybrid with an insertion between amino acids 99 and 100 (Fig. 7.16). Thus, as with the CstH::LT-B_{44-64} fusions pBC KS may be a better vector for the expression of hybrid proteins.

7.3 Discussion

In the previous chapter it has been shown that there are at least two sites in CstH which could accept the insertion of a 20 aa linear peptide (LT-B_{44-64}), without resulting

Fig. 7.14 Re-cloning of recombinant *cst* operons into pBC KS.

Plasmids pPM4571 and pPM4577 were digested with HindIII and the DNA fragments containing the recombinant *cst* operons were cloned into the *Hin*dIII site of pBC KS. The resultant plasmids were designated pPM4573 and pPM4579, respectively.

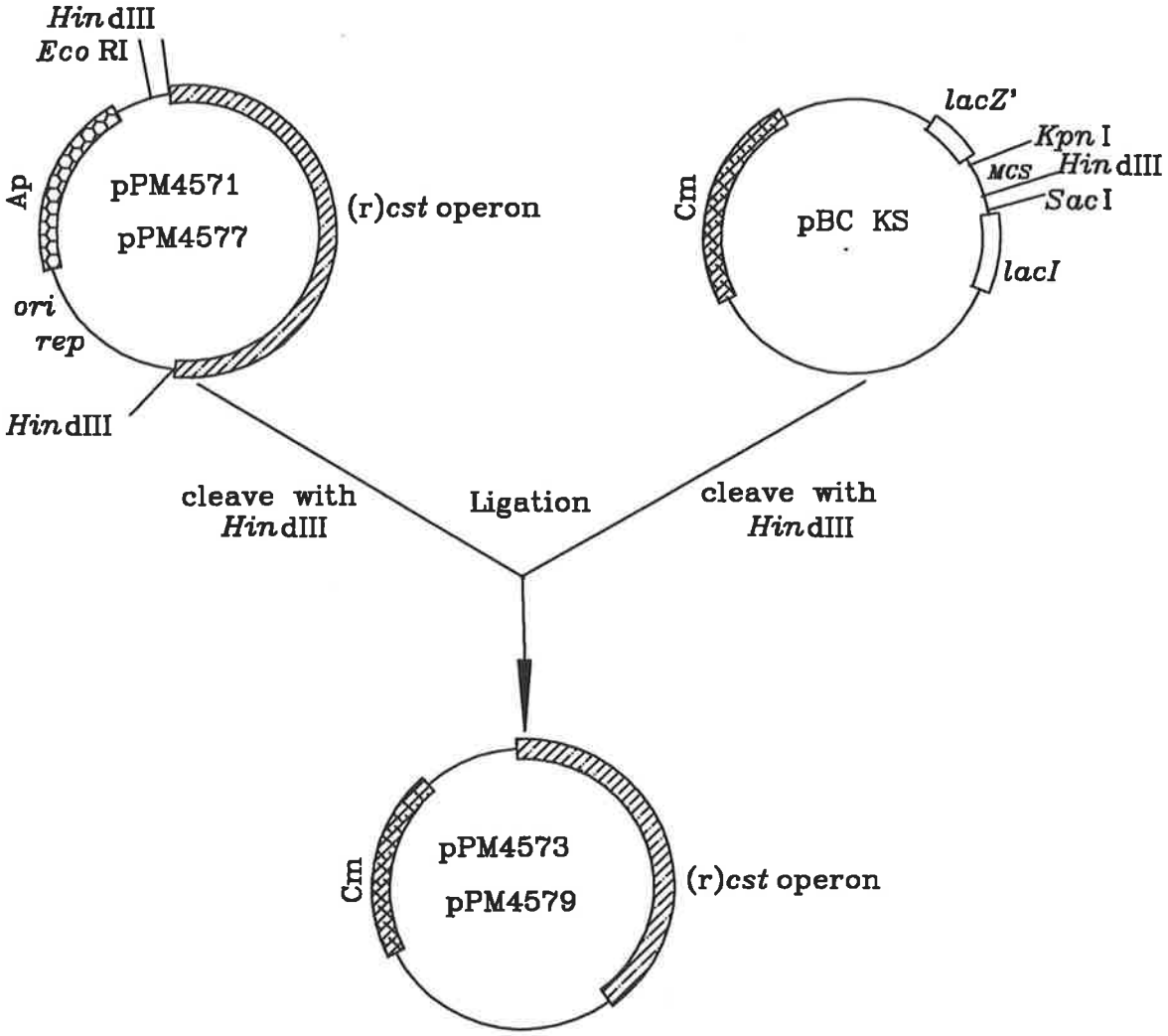

*Hin*dIII
Eco RI
Ap
pPM4571
pPM4577
(r)*cst* operon
ori
rep
*Hin*dIII
lacZ'
Kpn I
MCS
*Hin*dIII
Sac I
Cm
pBC KS
lacI
cleave with
*Hin*dIII
Ligation
cleave with
*Hin*dIII
Cm
pPM4573
pPM4579
(r)*cst* operon

Fig. 7.15 Western blot analysis of hybrid CstH proteins expressed by recombinant *cst* operons in pBC KS.

The CstH::ST hybrid proteins were expressed from recombinant *cst* operons and analysed in Western blot and detected by ECL. Whole cell lysates were electrophoresed in SDS on 15% polyacrylamide gels, transferred to a nitrocellulose filter, and probed with a polyclonal antiserum to CS3 (Panel A) and Mab CH10 to CstH (Panel B). The arrow corresponds to the position of CstH.

Lanes:

1. *E. coli* K-12[pPM484] expressing wild type CstH
2. *E. coli* K-12[pBC KS] vector plasmid for *cstH::est* (-control)
3. PB176 (+control)
4. *E. coli* K-12[pPM4579] expressing CstH::ST (between aa 99-100)
5. *E. coli* K-12[pPM4573] expressing CstH::ST (between aa 65-66)

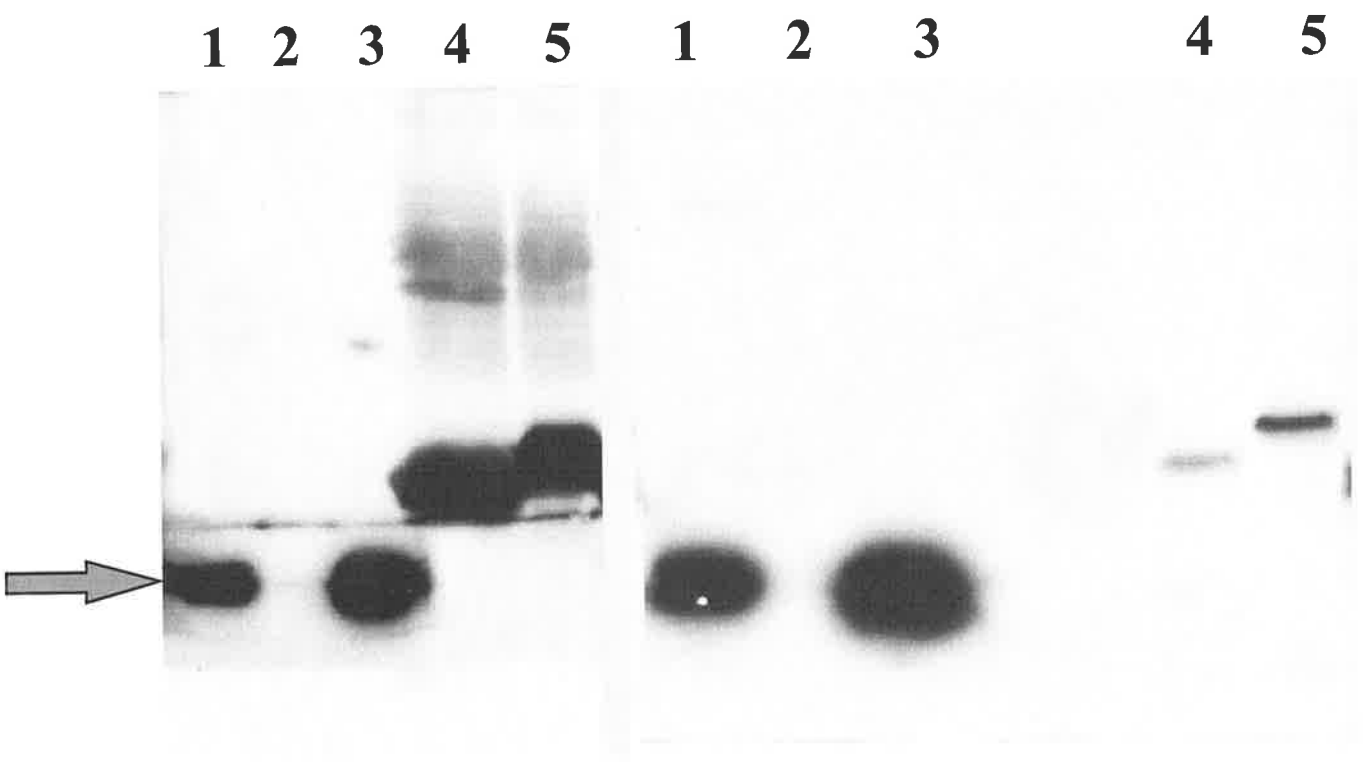
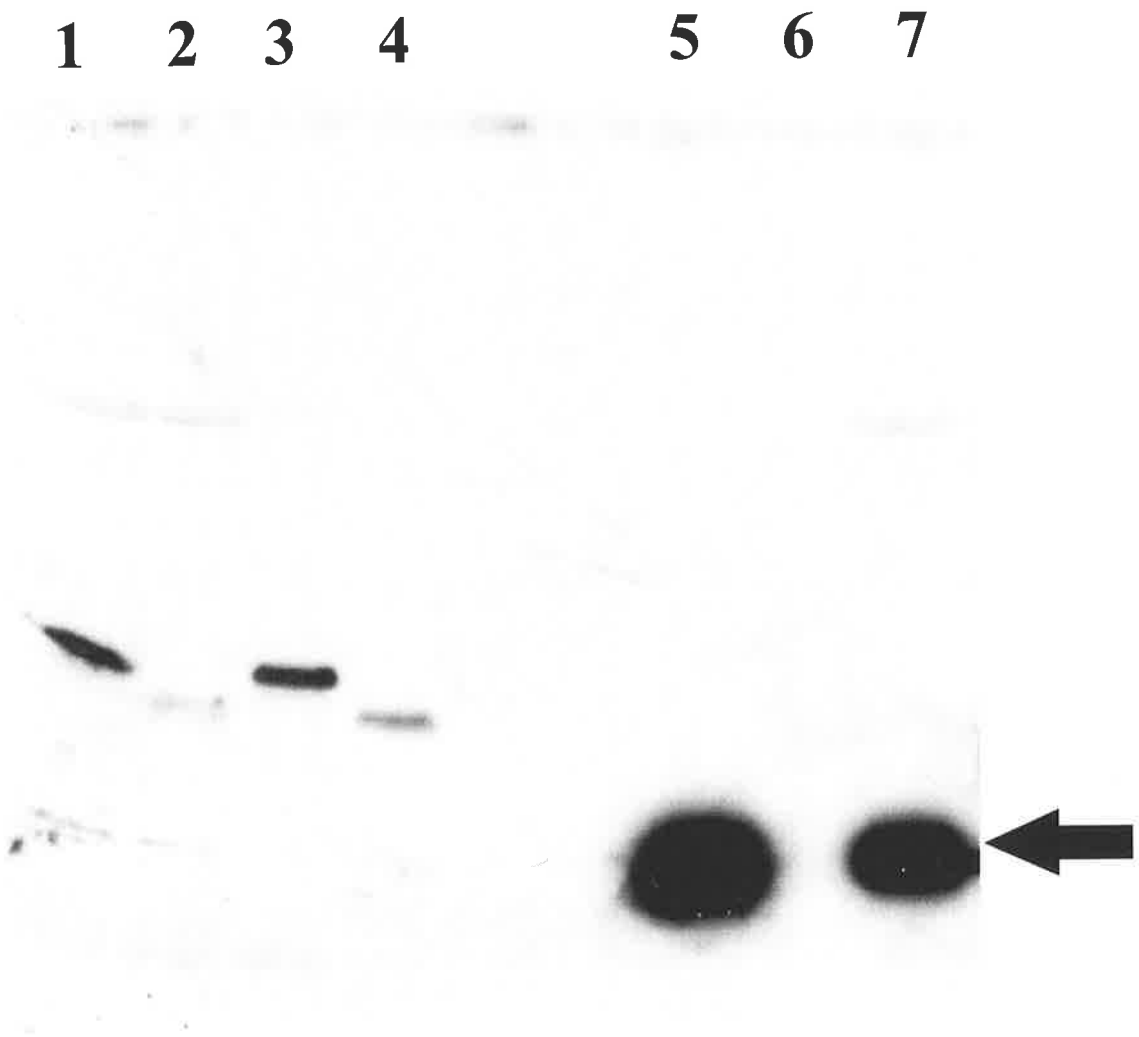

1 2 3 4 5 1 2 3 4 5

Fig. 7.16 Comparison of the expression of the CstH::ST proteins in different vectors

Expression of the hybrid CstH::ST proteins from recombinant *cst* operons in pBluescript (lane 1 and 2) and pBC KS (lanes 3 and 4) was analysed by Western blot. Whole cell lysates of 10^9 cells were electrophoresed in SDS on 15% polyacrylamide gels, transferred to a nitrocellulose filter, probed with Mab CH10 to CstH and visualized by ECL. The arrow corresponds to the position of CstH.

Lanes:

1. *E. coli* K-12[pPM4571] expressing CstH::ST (between aa 65-66)
2. *E. coli* K-12[pPM4577] expressing CstH::ST (between aa 99-100)
3. *E. coli* K-12[pPM4573] expressing CstH::ST (between aa 65-66)
4. *E. coli* K-12[pPM4579] expressing CstH::ST (between aa 99-100)
5. PB176 (+control)
6. *E. coli* K-12[pBluescript] vector plasmid for *cstH::est* (-control)
7. *E. coli* K-12[pPM484] expressing wild type CstH


1 2 3 4 5 6 7

in change to the overall antigenic structure of the CstH protein. The question arose whether this protein could tolerate the insertion of non-linear heterologous epitopes, especially peptides containing cysteine (Cys) residues. In theory, since there are no Cys in CstH (Jalajakumari *et al.,* 1989), the Cys residues of a foreign conformational epitope should not interfere with the folding of CstH, allowing it to function as a carrier for expression of this type of epitope. Thus, CstH has an advantage for the insertion of foreign conformational epitopes over other systems such as type 1 fimbriae in which the major fimbrial subunit, FimA, contains two Cys residues (Hedegaard and Klemm, 1989).

As mentioned in Chapters 1 and 6, several fimbriae and flagella have been used for insertion and expression of foreign epitopes. In most cases the epitopes are linear and do not contain Cys residues (Klemm and Hedegaard, 1990; Newton *et al.,* 1989; 1991; Van der Zee *et al.,* 1995; Bousquet *et al.,* 1994; Bakker, 1990). In two experiments conformational epitopes have been used for insertion. A 16 aa epitope of hepatitis B surface antigen containing two Cys residues has been inserted into the type 1 fimbrial subunit (FimA), but no fimbriae were produced. This may be a consequence of interference between the Cys residues in the epitope with those in FimA, preventing the assembly of FimA into fimbriae (Hedegaard and Klemm, 1989). It has also been shown that the K88ad subunit can accept a somatostatin-coding sequence containing two Cys residues and expressed hybrid pili around the cell. The hybrid pili were recovered from bacterial cultures, however when the same epitope was inserted in the K88ac subunit more hybrid pili were detected, suggesting that minor changes in the carrier protein can have significant effects on its utility (Thiry *et al*, 1989).

Mature ST was chosen as a complex peptide for insertion into CstH not only because of its structure but also for its importance in the pathogenesis of ETEC. ST contains six Cys residues, forming three disulfide bonds which are essential for the

tertiary structure and biological activity of the toxin (Yoshimura *et al.,* 1985; Gariepy *et al.,* 1987). The conformational epitopes of native ST elicit the production of antibodies against ST with neutralising and guanylate cyclase-inhibiting activity (Takeda *et al.,* 1993). In the other hand, ST is haptenic and only becomes immunogenic by coupling to high molecular weight carriers (Frantz and Robertson, 1981; Sanchez *et al.,* 1986; Clements, 1990).

By the insertion of mature ST into CstH and expression of the hybrid protein as pili, it may be possible not only to evaluate the flexibility of CstH for the acceptance of conformational epitopes, but it may also make the ST immunogenic and induce the production of antibodies against both ST and CstH. The hybrid proteins therefore may also be useful in developing a suitable vaccine against ST producing ETEC. Since several enteric bacteria produce ST, such hybrid proteins may also represent suitable components for inclusion in a multivalent vaccine against diarrhoeal diseases.

Like the CstH::LT-B_{44-64} fusions described in the previous chapter, there was little success in expressing the CstH::ST hybrids under the control of the CstH promoters using an *E. coli* K-12 *deg* ^{P} strain or in conjunction with the assembly genes. However, the hybrid proteins could be expressed using both the T7 promoter/RNA polymerase system and as hybrid *cst* operons. The higher level of expression of CstH hybrids from the hybrid CS3 operons may suggest that there are additional promoter or regulatory regions in the CS3 operon which are important for CstH expression. In both cases the CstH::ST hybrid proteins reacted with both antibodies against ST and CS3. These results demonstrate that CstH can accept the insertion of complex peptides or conformational heterologous epitopes containing Cys residues without any apparent affect on its antigenic structure. Thus, this may provide a system for the insertion and

expression of other conformational antigenic determinants whose expression in other systems is impossible or difficult.

Chapter 8

Characterization of hybrid CS3 pili

8.1 Introduction

In the previous chapters, the construction and reactivity of CstH::LT-B_{44-64} and CstH::ST hybrid proteins with antibodies have been described, along with their expression using the T7 promoter/RNA polymerase system and in conjunction with their natural CS3 biosynthetic system. In this chapter the expression of the hybrid proteins on the bacterial surface and their immunogenicity in mice will be evaluated.

Oral administration of vaccines, especially live attenuated *Salmonella* spp. is an effective means of inducing significant humoral and secretory antibody responses because the antigen is delivered to the Peyer's patch lymphoid follicles (Cardenas and Clements, 1992). A number of attenuated *Salmonella* spp. have been used for expression of heterologous antigens and considered as potential carriers for virulence factors of pathogens in vaccine development (Attridge *et al.,* 1988; Brown *et al.,* 1987; Curtiss *et al.,* 1987; Dougan *et al.,* 1987; Giron *et al.,* 1995; Maskell *et al.,* 1986; Morona *et al.,* 1994; Sadoff *et al.,* 1988; Stevenson and Manning, 1985; Xu *et al.,* 1993).

A galactose epimerase (*galE*) mutant of *Salmonella typhimurium* (strain G30) has been used for the expression of fimbrial antigens, and it has been demonstrated that immunization with hybrid *Salmonella/E. coli* strains produced high levels of secretory and humoral antibody responses to the heterologous antigens and immunity to the parent pathogen (Hone *et al.,* 1988; Morona *et al.,* 1994; Stevenson and Manning, 1985). In

the studies presented here, G30 has been used for the evaluation of the immunogenicity of hybrid CS3 pili in mice.

8.2 Results

8.2.1 Characterization of the hybrid CS3 pili

8.2.1.1 Polymerising capacity of the hybrid CstH proteins

The ability of the mutant CstH proteins to polymerise was checked by Western blot analysis using non-denaturing PAGE with a polyclonal antiserum to native CS3 pili. After expression of the mutant CstH and hybrid proteins in the T7 promoter/RNA polymerase system, cells were harvested, resuspended in SDS sample buffer lacking β-mercaptoethanol and used for the assessment of polymerisation. Western blot analysis (Fig. 8.1) of the non-denatured and denatured proteins revealed several immunologically related bands corresponding to the denatured CstH and CstH hybrid proteins as well as several bands which represent the polymerised form of these subunits. These results indicate that the mutations in *cstH* (corresponding to aa residues, 66-67, 100 and 109 of the mature protein) and the insertion of the foreign epitopes (in particular the ST insertion) into at least 2 regions of mutant CstH proteins (residues 65/66 and 99/100 of the mature protein) does not change the conformation and subunit-subunit binding sites which are responsible for polymerisation. Since pilus assembly requires subunit-subunit interaction for polymerisation, it is most likely that the recombinant CstHs are able to be polymerised in the periplasm and assembled as pili on the cell surface. To assess the expression and assembly of the recombinant CstHs, the recombinant *cst* operons with insertions of ST and LT-B_{44-64} between residues 65/66 and 99/100 of CstH were used in immunoblot and immunofluorescence microscopy experiments.

Fig. 8.1 Polymerisation of the mutant CstH proteins

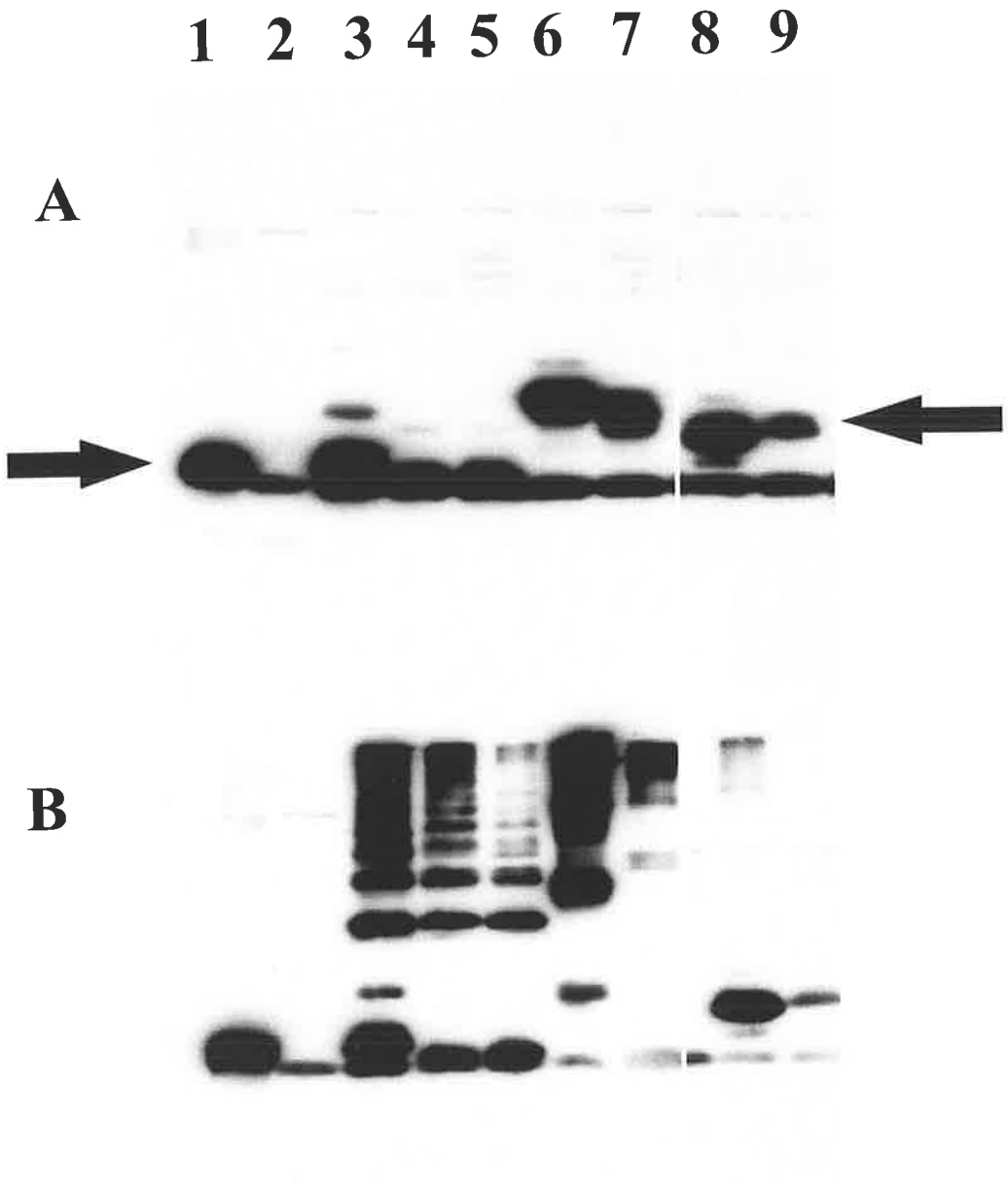
CstH and the mutant CstH proteins were analysed for polymerization by Western blotting and detected by ECL. Denatured (Panel A) and non-denatured (Panel B) samples (ca.10^9 cells) were electrophoresed in SDS on 15% polyacrylamide gels, transferred to nitrocellulose filters, probed with polyclonal antiserum to CS3 and visualized by ECL. The left arrow indicates the position of the CstH monomer and right arrow indicates hybrid CstH proteins.

Panel A: denatured proteins

1. PB 176 (+control)
2. PB 176p$^-$ (-control)
3. *E. coli* K-12[pPM4556] expressing mutant CstH with mutation in nt 4413-4417 of *cstH*
4. *E. coli* K-12[pPM4557] expressing mutant CstH with mutation in nt 4515-4518 of *cstH*
5. *E. coli* K-12[pPM4559] expressing mutant CstH with mutation in nt 4542-4545 of *cstH*
6. *E. coli* K-12[pPM4569] expressing CstH::ST (between aa 65-66)
7. *E. coli* K-12[pPM4576] expressing CstH::ST (between aa 99-100)
8. *E. coli* K-12[pPM4585] expressing CstH::LT-B_{44-64} (between aa 65-66)
9. *E. coli* K-12[pPM4590] expressing CstH::LT-B_{44-64} (between aa 99-100)

Panel B

The samples are the same as in panel A. Non-denatured proteins were prepared by dissolving the bacteria in SDS-sample buffer lacking β-mercaptoethanol and loaded onto the gel without boiling.


1 2 3 4 5 6 7 8 9
A
B

8.2.1.2 Expression of the hybrid CS3 pili

Expression of the hybrid pili on the bacterial surface was investigated by immunodot blot analysis and immunofluorescence microscopy using Mabs against CstH and recombinant *cst* operons in the plasmid vector pBC KS. These clones were chosen for expression of hybrid CS3 pili due to the higher level of expression of the hybrid proteins compared to their expression in pBluescript (see sections 6.2.7 and 7.2.5).

Bacteria harbouring plasmids which encode CstH::LTB_{44-64} (pPM4713 and pPM4715) and CstH::ST (pPM4573 and pPM4579) hybrid proteins were grown overnight on CFA containing Cm. Cells were harvested in PBS and the pili prepared (section 2.18.1.1) and used for immunodot blot analysis. Both the polyclonal antiserum to CS3 and Mab against CstH were able to detect the hybrid pili. The reactivity of some Mabs with hybrid CS3 pili has been shown in Fig. 8.2.

The Mabs against CstH and ST could also recognize the hybrid pili on the surface of these bacteria by immunofluorescence microscopy (Fig 8.3 and 8.4, respectively).

These results show that CstH can tolerate insertion of heterologous peptides, even complex peptides such as ST with six Cys residues, and can be assembled as a pili on the surface of the bacteria.

8.2.1.3 Effect of the insertions on the antigenic property of CS3

The effect of the insertions of heterologous peptides, with regard to both the insert and the insertion site, on the structure and antigenicity of CS3 pili was investigated using a panel of Mabs and hybrid pili.

The hybrid pili from bacteria expressing CstH::LT-B_{44-64} and CstH::ST hybrid proteins encoded on plasmid pPM4713, pPM4715, pPM4573 and pPM4579 were

Fig. 8.2 Immunodot blot analysis of hybrid CS3 pili

Pili were prepared from the bacteria and blotted onto the nitrocellulose filter and their reactivity with a polyclonal antiserum to CS3 (Panel A) and to various Mabs directed against CstH (Panel B) were visualized by ECL.

Panel A:

1. *E. coli* K-12 carrying pPM484
2. *E. coli* K-12[pPM4571] expressing CstH::ST (between aa 65-66)
3. *E. coli* K-12[pPM4577] expressing CstH::ST (between aa 99-100)
4. *E. coli* K-12[pPM4588] expressing CstH::LT-B_{44-64} (between aa 65-66)
5. *E. coli* K-12[pPM4593] expressing CstH::LT-B_{44-64} (between aa 99-100)
6. *E. coli* K-12[pPM4573] expressing CstH::ST (between aa 65-66), in pBC KS
7. *E. coli* K-12[pPM4579] expressing CstH::ST (between aa 99-100), in pBC KS
8. *E. coli* K-12[pPM4713] expressing CstH::LT-B_{44-64} (between aa 65-66), in pBC KS
9. *E. coli* K-12[pPM4715] expressing CstH::LT-B_{44-64} (between aa 99-100), in pBC KS

a: pili from 10^8 cells b: pili from 5×10^7 cells c: pili from 10^7 cells

Panel B

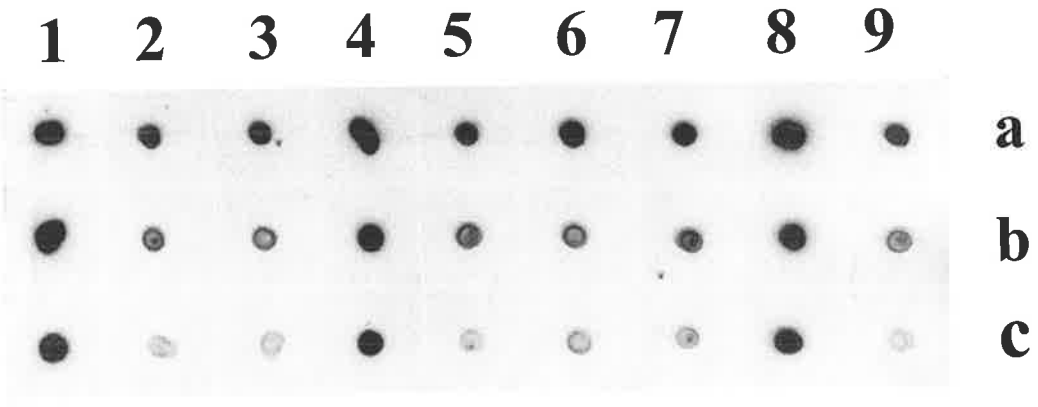
Purified pili from 10^8 cells were analysed as in panel A, but using a number of different Mabs.

1. Purified CS3 (+control)
2. *E. coli* K-12[pBC KS]vector plasmid for hybrid CstH (-control)
3. *E. coli* K-12[pPM4573] expressing CstH::ST (between aa 65-66), in pBC KS
4. *E. coli* K-12[pPM4579] expressing CstH::ST (between aa 99-100), in pBC KS
5. *E. coli* K-12[pPM4713] expressing CstH::LT-B_{44-64} (between aa 65-66), in pBC KS
6. *E. coli* K-12[pPM4715] expressing CstH::LT-B_{44-64} (between aa 99-100), in pBC KS

d: polyclonal antibody

e-j: Mabs CH1-CH6 against CstH

A



B

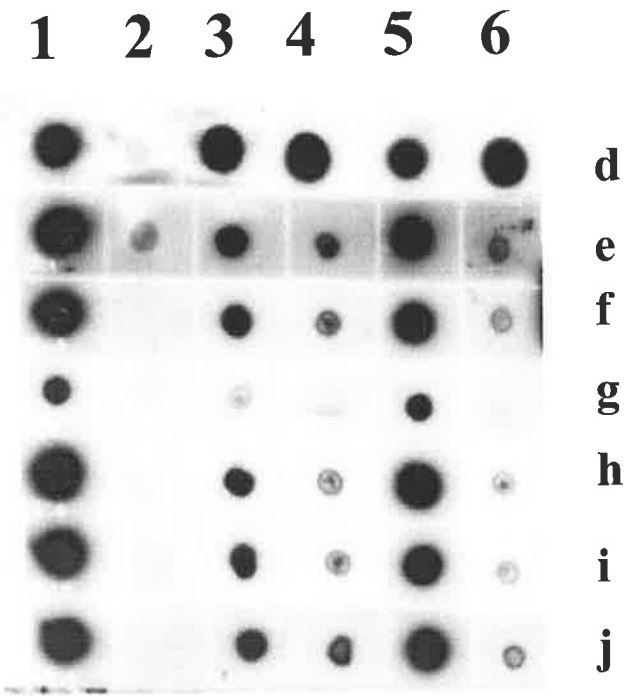


Fig 8.3 Immunofluorescence detection of hybrid CS3 pili.

Bacteria harbouring expressing hybrids CstH::ST and CstH::LT-B_{44-64} were fixed on glass slides, treated with Mab to CstH and visualised under the fluorescence microscope.

A. *E. coli* K-12[pPM4573] expressing CstH::ST (between aa 65-66), in pBC KS

B. *E. coli* K-12[pPM4579] expressing CstH::ST (between aa 99-100), in pBC KS

C. *E. coli* K-12[pPM4713] expressing CstH::LT-B_{44-64} (between aa 65-66), in pBC KS

D. *E. coli* K-12[pPM4715] expressing CstH::LT-B_{44-64} (between aa 99-100), in pBC KS

Negative control PB176p$^-$ is not shown because no fluorescent bacteria were able to be detected.

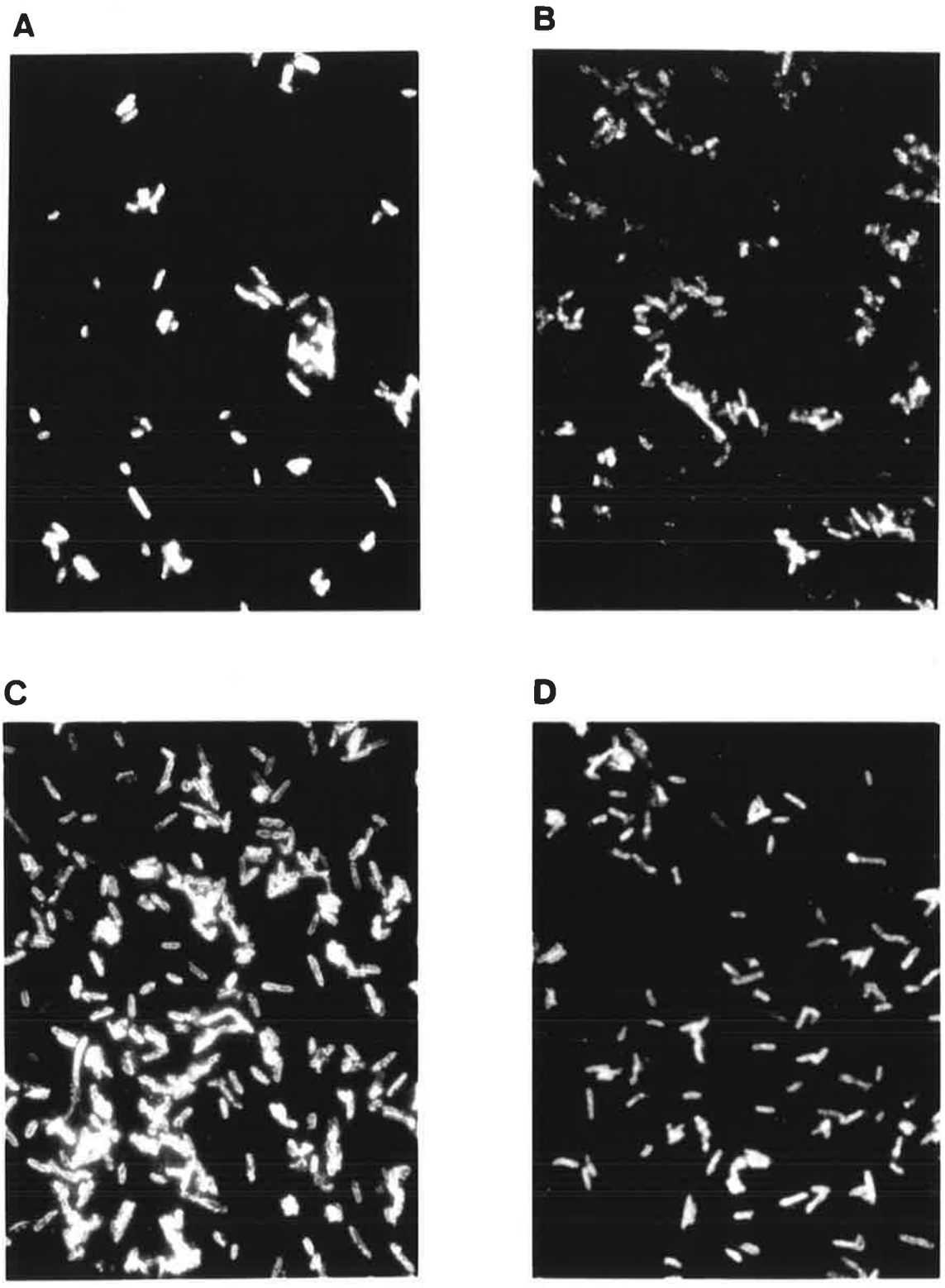
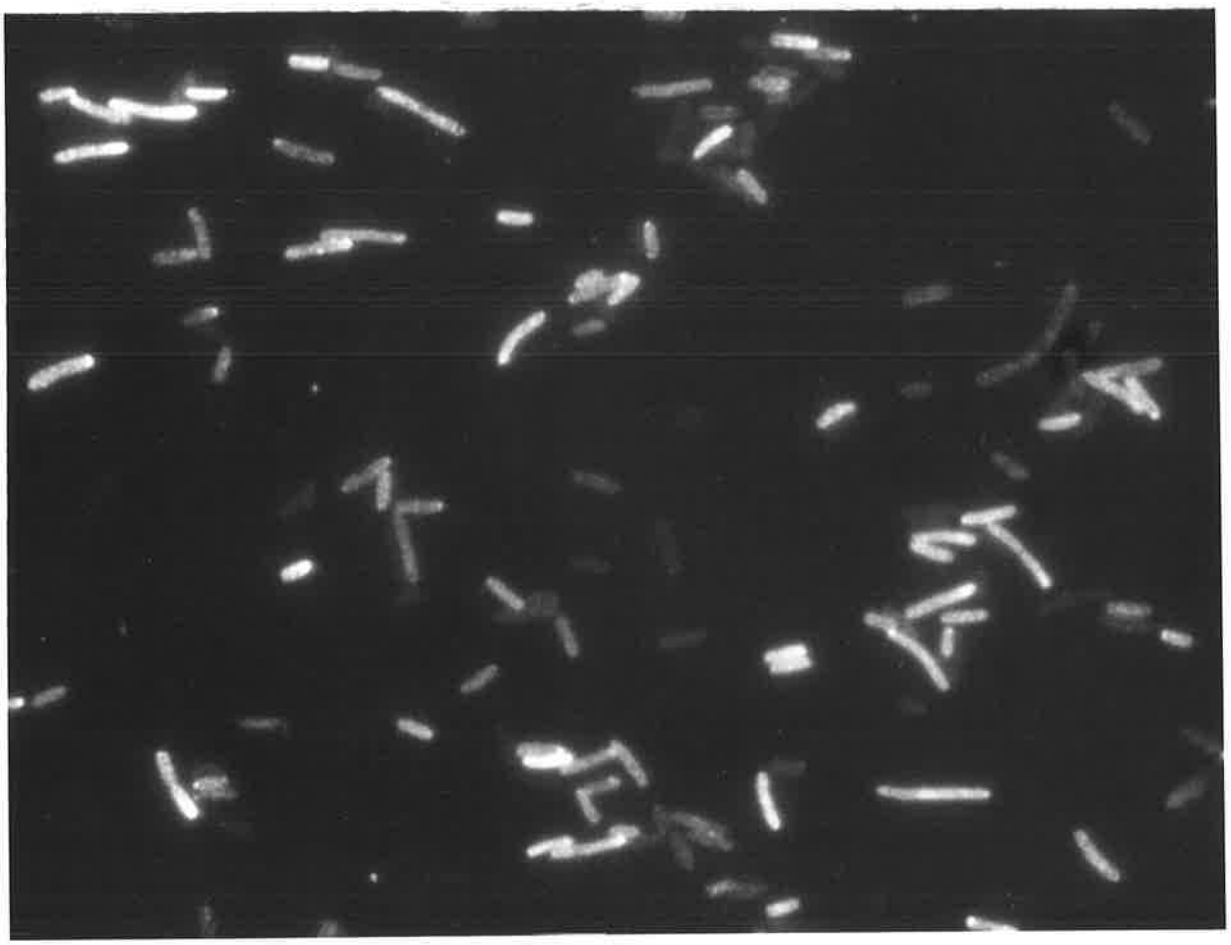

A
B
C
D

Fig 8.4 Immunofluorescence detection of hybrid CS3 pili using Mab to ST.

Bacteria harbouring plasmid pPM4573 expressing hybrids CstH::ST (between aa 65-66) were fixed on glass slides, treated with Mab to ST and visualised under the fluorescence microscope.



prepared and used in immunodot blot analysis. Pili prepared from 10^8 cells were blotted onto nitrocellulose paper, treated with either the polyclonal antiserum to CS3 or the Mabs against CstH, and the antibody-antigen complexes detected by ECL. The density times area of the blots was measured on the Analytical Imaging Station (Imaging Research Inc).

Two sets of Mabs were used in immunodot blot analysis. Mabs CH1-CH19, 10:2 and 11:2 are produced by cloned hybridomas. Mabs Cst1-Cst16 are supernatants from cell cultures which have not been cloned.

The polyclonal antiserum against CS3 could recognize all hybrid pili (Fig. 8.2). The Mabs had different reactivity with hybrid pili. The density times area for the blots was in the range of 0-150 above the background (-control). The distribution of the values obtained with the Mabs is shown in Table 8.1. The Mabs have been classified into several groups according to their reactivity with the hybrid pili (Table 8.2). Mabs were classified in four groups: Mabs with scoring below 5 were considered negative and above it, positive. Mabs with values of 5-35 were indicated by +, 36-70 by ++ and above 70 by +++. The positive controls always had the density times area above 70.

All Mabs, except CH9 and CH11, could bind to the hybrid CS3 with the insertion of the LT-B epitope between amino acids 65-66 of mature CstH implying that these Mabs are not directed against this region. However, Mabs CH9 and CH11 reacted with the hybrid proteins with ST in the same site, implying that these Mabs may not be directed against this region, but their binding can be influenced by conformational changes at this site. The epitope(s) for Mabs in groups 8, 11, 12 and 13 is probably close to this site and thus the insertion has not disrupted the epitope completely, allowing the Mabs still bind, albeit weakly.

Table 8.1 Recognition of CstH::LT-B_{44-64} and CstH::ST hybrid proteins by different Mabs.

Density times area	Number of Mabs to CstH reacted with pili from *E. coli* K-12 carrying pPM4713	pPM4715	pPM4573	pPM4572
0-5	2	23	13	21
6-10	3	3	3	4
11-15	1	1	3	5
16-20	2	3	2	1
21-25	0	5	3	2
26-30	4	1	1	2
31-35	0	0	3	1
36-40	0	0	6	1
41-45	0	0	3	0
46-50	1	0	0	0
51-55	0	1	0	0
56-60	0	0	0	0
61-65	1	0	0	0
66-70	2	0	0	0
71-75	1	0	0	0
76-80	1	0	0	0
81-85	1	0	0	0
86-90	6	0	0	0
91-95	0	0	0	0
95-100	1	0	0	0
101-105	1	0	0	0
106-110	3	0	0	0
111-115	1	0	0	0
116-120	2	0	0	0
121-125	0	0	0	0
126-130	2	0	0	0
131-135	0	0	0	0
136-140	1	0	0	0
141-145	0	0	0	0
146-150	1	0	0	0

Table 8-2 Classification of Mabs based on their reactivity with hybrid CstH proteins encoded by plasmids

Groups	Mabs	pPM4713	pPM4715	pPM4573	pPM4579
1	Cst1	+++	++	+	+
2	Cst13	+++	+	++	++
3	Cst4, Cst7, Cst18	+++	+	++	+
4	CH1	+++	+	++	-
5	CH2, CH6, CH8, Cst6	+++	-	++	+
6	CH7, 10:2, Cst8, Cst10	+++	+	+	+
7	Cst2, Cst14, Cst15,	+++	+	+	-
8	Cst11	++	+	+	+
9	Cst17	+++	-	-	-
10	CH4, CH5, 11:2	+++	-	+	-
11	CH10, Cst3, Cst9	++	-	-	-
12	CH19	+	-	+	-
13	CH3, CH12, CH13, CH14, CH15, CH16, CH17, CH18, Cst5	+	-	-	-
14	CH9, CH11	-	-	+	-

Cst1-Cst18: Culture supernatants from cultures Cst1-Cst18

CH1-CH19: Mabs CH1-CH19

pPM4713, pPM4715 encoding CstH::LT-B_{44-64} with insertion between aa# 65-66 and 99-100 respectively

pPM4573 and pPM4579 encoding CstH::ST with insertion between aa # 65-66 and 99-100 respectively

Insertion of ST in the same site had a more dramatic effect on the antigenic properties of CstH and all Mabs had weaker reactivity with the hybrid proteins. The epitope for the Mabs in groups 9, 11, 13 and 14 may be adjacent to this site or alternatively, the epitope is conformational with a contribution from this area and the insertion of ST (and LT-B_{44-64} in the case of group 14) disrupting the conformational epitope.

Insertion between amino acids 99 and 100 of mature CstH had a greater effect on the ability of CS3 to be recognized by the Mabs and the binding was abolished for Mabs in groups 4 5,7, 9, 10, 11, 12, 13 and 14. This suggests that this region is an epitope or part thereof for these Mabs or alternatively, the epitope for Mabs in group 5 and 7 is close and has been disrupted by the insertion of ST . Mabs Cst2, Cst14 and Cst15 (group 7) which reacted with CstH::LT-B_{44-64} could not recognize CstH::ST and Mabs CH2, CH8, Cst6 (group 5) and CH6 did not react with CstH::LT-B_{44-64} but reacted with CstH::ST.

In general, the epitopes for Mabs in groups 1, 2 and 3 are likely to be located distant from both insertion sites. Mabs in groups 6, 7 and 8 could be in a region between the two sites or in an area which could be affected by insertion at both sites. The epitope for Mabs in groups 7, 10, 12 and 14 may be located closer to amino acids 99 and 100 or be more affected by insertion at this site. The epitopes for groups 9, 11 and 13 could be located in a region which includes amino acids 99 and 100 and close to the insertion site of amino acids 65/66 in the three dimensional structure of the protein, since insertion in site 99-100 abolished the binding of the Mabs and the binding was also affected by insertion in the site 65-66.

These results suggest that:

1- The different Mab classes recognize different epitopes in CS3.

2- Insertion of ST has the more pronounced effect on the antigenic structure of CstH. However, these insertions did not adversely affect the structure of CstH as the hybrid proteins can still be assembled into pili and the hybrid pili can be recognized by the polyclonal antiserum to intact CS3 and some Mabs.

3- The region that includes amino acids 99-100 is more sensitive to insertion of heterologous peptides, but the site between amino acids 65-66 can accept the insertion of foreign epitopes.

4- The epitopes of CstH, at least most of those recognized by the Mabs here, are conformational and the insertion of a more complex peptide has greater effect on the structure of these epitopes.

8.2.2 Immunogenicity of hybrid CS3 pili

8.2.2.1 Expression of hybrid CS3 pili in the *Salmonella* vaccine strain

The avirulent *Salmonella typhimurium* strain G30 (galactose epimerase-less, *galE* mutant of strain LT2, Osborn *et al.*, 1964) was used for delivery of the wild type and the recombinant CS3 pili to the immune system of mice. Hybrid pili with an insertion between amino acids 65/66 were chosen for immunization due to the higher level of expression and the apparently lower effect on the CstH epitopes. Most of the Mabs could react with these hybrid CstH proteins and could recognize the hybrid pili in immunodot blot analyses. Plasmids pPM4568, pPM4573 and pPM4713 encoding CS3 and hybrid CS3 pili (CstH::ST and CstH::LT-$B_{44\text{-}64}$, respectively) were transformed into strain LB5010 (Bullas and Ryu, 1983), the DNA re-isolated and then transformed into *S. typhimurium* G30. This transition of DNA through strain LB5010 overcomes the

problem of restriction of the transforming DNA by strain G30. The transformants were selected on to Cm and screened for the presence of the correct plasmid DNA.

Expression of the CstH hybrid proteins and the hybrid CS3 pili on the cell surface was confirmed by immunoblot analysis using polyclonal antiserum to CS3 (Fig 8.5) and immunofluorescence microscopy using Mab to CstH (Fig8.6). The resulting strains G30[pPM4568], G30[pPM4573] and G30[pPM4713] were used for immunization of the mice.

8.2.2.2 Immunization experiment

Strain G30 harbouring plasmids encoding CS3 and hybrid CS3 pili were grown overnight on CFA containing Cm (to select for maintenance of the plasmid). Bacteria were harvested in saline and used for oral immunization. Aliquots of the same cells were also treated with 0.1% formalin to prepare killed bacteria for booster injection.

Groups of 5 mice were orally immunized with 10^9 live bacteria in 30µl saline after having first been fed 30µl of 10% saturated $NaHCO_3$. On day 9 and 16, the mice were injected intraperitoneally with 10^7 formalin-killed bacteria. This regimen has been shown to induce the production of the secretory and humoral antibody against K88 pili of *E. coli* delivered by the G30 strain (Stevenson and Manning, 1985). Five days after the last immunization blood was taken and the sera pooled. After euthanasia with CO_2, the intestines were removed from the mice and washed with saline.

Sera and gut washings obtained from mice immunized with strains G30[pPM4568], G30[pPM4573] and G30[pPM4713] were used to test for the presence of antibodies against CS3, ST and LT-B in an ELISA using purified CS3, ST (Sigma) and LT (Sigma) as coating antigens. The titre of antibody was defined as the dilution of sera or gut washing which gave an A_{405} of 0.4 above the background (normal sera). The

Fig 8.5 Analysis of the expression of the hybrid CS3 pili in *S.typhimurium* G30

Expression of the CstH hybrid proteins from recombinant *cst* operons in *S.typhimurium* G30 was analysed by Western blot. Whole cell lysates were electrophoresed in SDS on 15% polyacrylamide gels, transferred to a nitrocellulose filter, probed with a polyclonal antiserum to CS3 and visualized by ECL. The left arrow indicates the position of CstH and the right indicates the CstH hybrid proteins.

Lanes:

1. *S.typhimurium* G30[pPM4573] encoding CstH::ST (between aa 65-66)
2. *S.typhimurium* G30[pPM4579] encoding CstH::ST (between aa 99-100)
3. PB176 (+control)
4. *S.typhimurium* G30/vector plasmid for *cstH::est* (-control)
5. *E. coli* K-12[pPM484] expressing wild type CstH
6. *S.typhimurium* G30[pPM4713] encoding CstH::LT-B_{44-64} (between aa 65-66)
7. *S.typhimurium* G30[pPM4715] encoding CstH::LT-B_{44-64} (between aa 99-100)

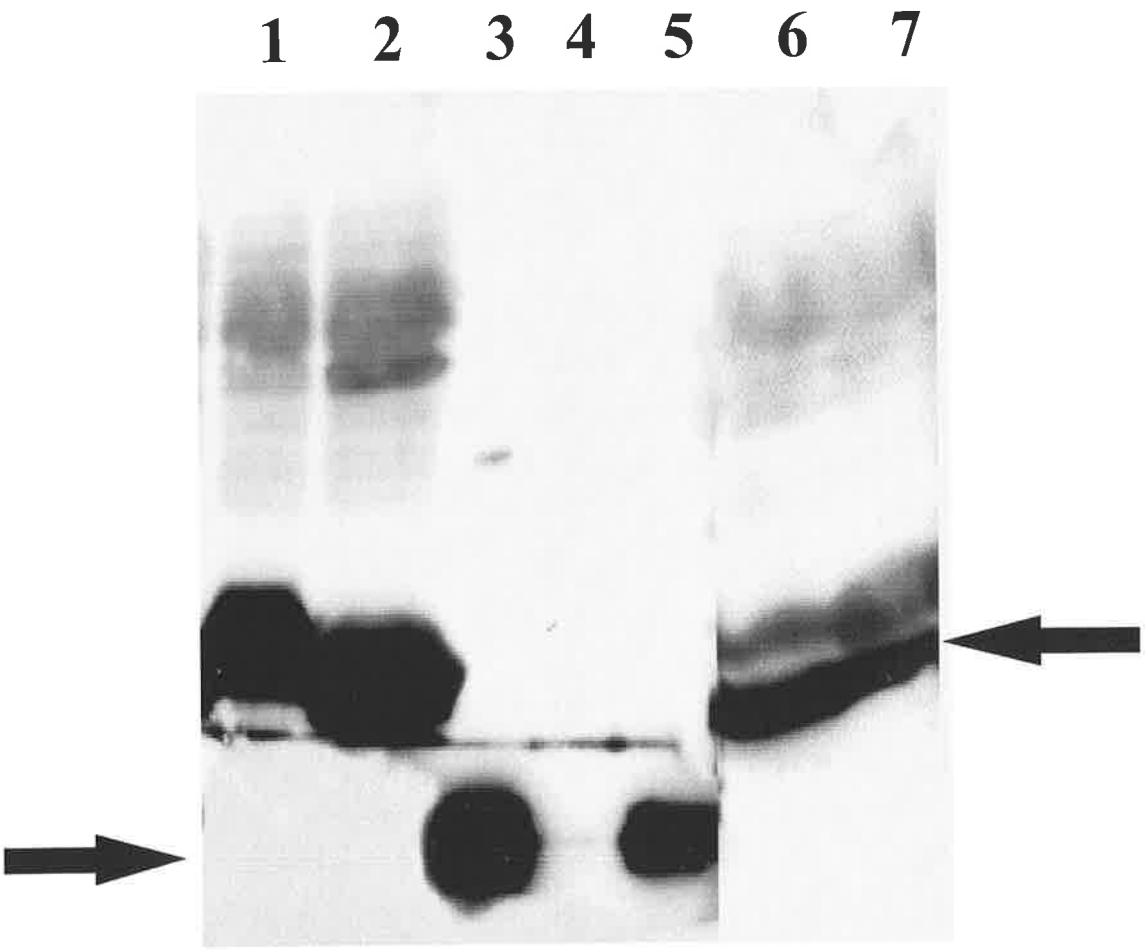

1 2 3 4 5 6 7

Fig. 8.6 Immunofluorescence detection of hybrid CS3 pili on *S.typhimurium* G30

Bacteria harbouring plasmids encoding hybrid CstH proteins were fixed on glass slides, treated with Mab CH10 to CstH and visualised under the fluorescence microscope.

A. *S.typhimurium* G30[pPM4573] encoding CstH::ST (between aa 65-66)

B. *S.typhimurium* G30[pPM4579] encoding CstH::ST (between aa 99-100)

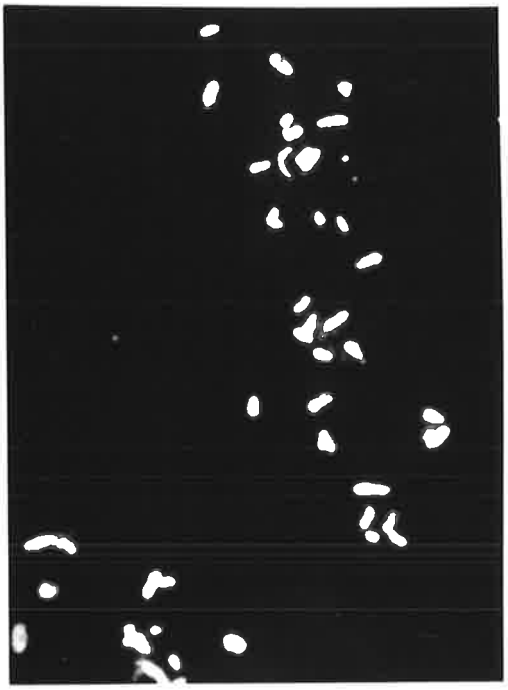
C. *S.typhimurium* G30[pPM4713] encoding CstH::LT-B_{44-64} (between aa 65-66)

D. *S.typhimurium* G30[pPM4715] encoding CstH::LT-B_{44-64} (between aa 99-100)

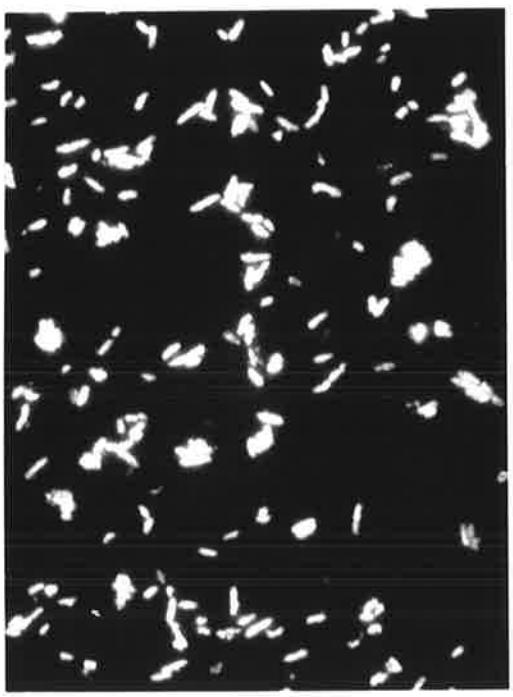
The negative control *S.typhimurium* G30 is not shown since no fluorescent bacteria were able to be detected.

A

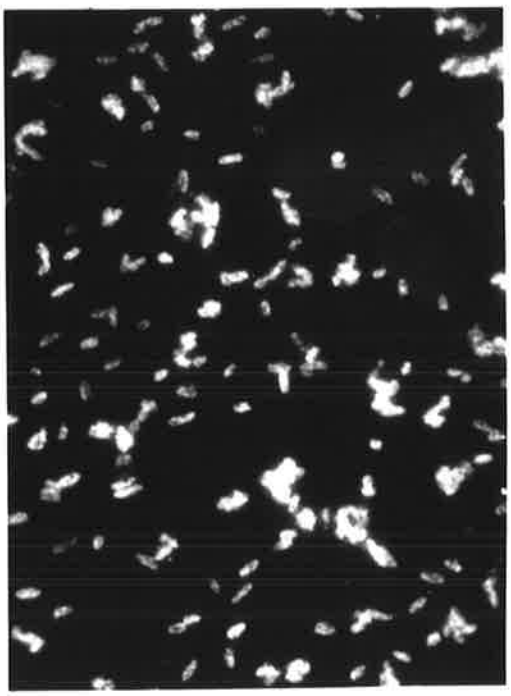
B



C



D



results indicated that the sera contained antibody subclass IgG to CS3 with the titres of 6×10^4, 800 and 100 for sera from mice immunized with G30 expressing CS3, CstH::ST and CstH::LT-B_{44-64} pili, respectively. IgA was not detected in these sera. The gut washings from mice immunized with G30[CS3], G30[CstH::ST] and G30[CstH::LT-B_{44-64}] showed the presence of IgG with titres of 16, 10 and 8 and IgA with the titre of 4, 6 and 2, respectively (Table 8.3). In ELISA using ST and LT as antigens, no anti-ST or anti-LT could be detected in sera and gut washings.

8.3 Discussion

Expression of heterologous antigens such as fimbriae on the surface of bacteria, especially vaccine strains such as attenuated *Salmonella* spp, has been considered a useful approach in vaccine development (Attridge *et al.,* 1988; Giron *et al.*, 1995; Morona *et al.,* 1994; Stevenson and Manning, 1985; Xu *et al.,* 1993). In addition, the expression of foreign epitopes on cell appendages like fimbriae has also been considered a worth-while adjunct to such programmes (Bakker *et al.,* 1990; Bousquet *et al.,* 1994; Van der Zee *et al.,* 1995; Van Die *et al.,* 1990). In order to test the suitability of CS3 fimbriae for these systems, an attempt has been made to construct hybrid proteins (CstH::LT-B_{44-64}, Chapter 6; CstH::ST, Chapter 7) and express them on the surface of bacteria for vaccine development purposes.

In this chapter, the hybrid proteins were characterized and the expression of the hybrid pili on the surface of *E. coli* K-12 strain DH5α and *Salmonella typhimurium* strain G30 and their immunogenicity has been presented. In addition, the effect of the insertion on the antigenicity of CstH was studied. Although the insertion of heterologous peptides did not alter the polymerising capacity of CstH, and the hybrid CstH proteins could be assembled as pili, the insertion of these peptides caused minor

Table 8.3 CstH antibody responses after immunization with hybrid CstH::LT-B_{44-64} and CstH::ST

Sera from mice immunized with	IgG titre of sera	IgG titre of gut wash	IgA titre of gut wash
Normal sera	0	0	0
S.typhimurium G30[pPM4568] (CS3)	4×10^4	16	4
S.typhimurium G30[pPM4713] (CstH::LT-B_{44-64})	100	8	2
S.typhimurium G30[pPM4573] (CstH::ST)	800	10	6

changes in the structure and disrupted some epitopes of CstH. As a result, some of the Mabs did not react with the hybrid proteins. This property was also affected by the nature of the insert and insertion site. Insertion in between amino acids 99 and 100 decreased the expression of hybrid proteins and some Mabs did not react with hybrid proteins which may show that this region is an epitope of CstH which can be disrupted by the insertion of foreign epitopes. The area that includes amino acids 65 and 66 was more resistant to the effects of insertion. Hybrid proteins with insertions at this site were expressed better, although at lower levels than wild type CS3, and most of the Mabs could recognize the hybrid pili. Insertion of ST caused more changes in CstH and more Mabs did not recognize the hybrid proteins.

The results presented in this study suggest that CstH can accept insertion of heterologous peptides and still be assembled as pili on the surface of the bacteria. The hybrid pili were expressed on *E. coli* K-12 from the natural promoters, albeit at low levels. In the case of Ap^R plasmids, the low level of expression of hybrid pili could be attributed to the instability of the plasmids and the overgrowth by the bacteria lacking plasmids encoding hybrid pili. This problem was partially overcome by recloning the recombinant *cst* operons in pBC KS which confers Cm^R. Under these conditions, good expression of hybrid CS3 was obtained as measured by immunoblot analysis. The hybrid pili were also expressed in *Salmonella typhimurium* strain G30 as shown by Western blot analysis and immunofluorescence microscopy.

G30 has been used for expression of K88 and K99 fimbriae, immunization and raising antibodies against ETEC (Attridge *et al.,* 1988; Morona *et al.,* 1994; Stevenson and Manning, 1985). In the experiments described here it has been possible to demonstrate the induction of antibodies against fimbriae, but with CstH::ST and CstH::LT-B_{44-64} chimeric pili, only antibodies to CS3 could be detected by ELISA using

sera and gut washings from immunized mice. No antibodies to ST or LT could be detected in sera or gut washings. Immunofluorescence microscopy showed the binding of Mab against ST to the bacteria expressing hybrid CS3::ST which demonstrates exposure of the foreign epitope. It is clear that the plasmids encoding hybrid CS3 are highly unstable *in vitro* and this is likely to be problematic *in vivo* as well, in fact this may be reflected by the 100-fold weaker anti-CS3 response obtained with the hybrid pili compared to the unmodified CS3 (as shown by ELISA). The instability of the recombinant plasmids suggests that the plasmid may have been lost in oral inocula and negligible amounts of antigen were presented to the immune systems of the animals. Since fimbriae are immunogenic antigens, the low level presented has not precluded the production of detectable levels of anti CS3 antibodies. However, if the foreign epitopes are not as immunogenic, then the production of anti ST and LT-B antibodies could be below detectable levels. It is also possible that only the booster injection with killed bacteria elicited the production of antibodies mainly against the body of the hybrid pili. The failure of the production of antibodies to the foreign epitopes may also be due to masking of them by CstH, which prevents the presentation of heterologous epitopes to the animal immune system. The possible reasons for the failure of the animal to respond to ST should be investigated in future studies.

Chapter 9

General discussion

9.1 Introduction

Infectious diseases are still a world wide problem with diarrhoea due to enterotoxigenic *E. coli* (ETEC) being a major contributor. ETEC is the major cause of diarrhoea in developing countries and in travellers (Black, 1993) with about 50 million cases and 800000 deaths annually (Black, 1986). There are two classes of pathogenic factors associated with ETEC. These are adhesins referred to as colonization factor antigens, pili or fimbriae, and the toxins. Several colonization factor antigens (CFA) including CFA/I, CFA/II, CFA/III and CFA/IV and putative colonization factors (PCF) have been identified on ETEC. The toxins which are elaborated from bacteria attached to the intestinal brush border are heat labile (LT) and heat stable (ST) and stimulate accumulation of water and electrolytes in the intestine leading to diarrhoea. The most effective long-term solution to control the disease is improved hygiene and the provision of safe water supplies. A short-term solution is the development of an effective vaccine particularly for travellers to the endemic regions. There have been many efforts to generate a vaccine against ETEC. However, since ETEC express several different colonization factors, and in addition two toxins, an effective vaccine should protect against as many virulence factors as possible. Some efforts has been made to produce bivalent vaccines by fusion of ST or its related peptides to LT-B or a part thereof through chemical procedures or recombinant DNA technology. Fimbriae have also been used to elicit antibodies against ETEC. However, as yet no effective vaccine is available

for human use. On the other hand, bacterial surface molecules and appendages such as fimbriae have been used for expression of antigenic determinants of pathogens with the purpose of vaccine development.

The major aim of this project was development of a delivery system for expression of heterologous antigenic determinants based on the CS3 subcomponent of the colonization factor antigen II (CFA/II) of ETEC with the aim to generate a vaccine or component of a vaccine against ETEC. The approaches taken involved studying the antigenic variation in CS3, mapping exposed epitopes, insertion of ST and a part of LT-B as foreign epitopes in CstH, expression of hybrid proteins as hybrid pili on the surface of *E. coli* K-12 and *Salmonella typhimurium* G30 and immunization of mice.

9.2 Isolation and characterization of Mabs against CstH

A panel of Mabs was produced in order to study the antigenic variation among CS3 from different geographical areas and for epitope mapping of CstH. 19 cell cultures producing Mabs against CstH were prepared. After cloning this resulted in 25 monoclonal antibody producing cell lines including 19 Mabs against CstH and 6 against LPS.

The anti-CstH Mabs were able to recognize CS3 pili in six ETEC strains expressing different combinations of CS factors by immunodot blot and immunofluorescence microscopy. Since CS3 is expressed by most CFA/II$^+$ ETEC, these Mabs may be useful in epidemiological studies to define the prevalence of the CFA/II$^+$ involved in disease in different geographical regions and so evaluate its importance for vaccine development in these areas.

These Mabs are against several different epitopes of CstH as demonstrated by the variation in reactivity with CstH::ST and CstH::LT-B_{44-64} hybrid proteins. However, the

exact sequences of the epitopes recognized by the Mabs were not identified because the Mabs appear to recognize conformational epitopes. They may also be useful in studies related to the structure of the CstH and subunit-subunit binding in CS3, as well as the biogenesis and assembly of the CS3 pili.

The cross-reactivity of the Mabs with other CS factors has not been investigated but this could be another aspect of future work. If there are any shared or common epitopes among the colonization factor antigens, they may be useful in vaccine development against these bacteria provided they inhibit colonization. The unique epitopes, are also applicable to diagnostic and epidemiological studies.

The Mabs directed against LPS have not been studied in more detail but the preliminary data presented here suggests that they recognize the O-antigen component. Future investigation could characterize these Mabs and they may have application to the study of LPS or its biogenesis in *E. coli* or other Enterobacteriacae.

The protective capacity of the Mabs can also be evaluated in animal models by mixing the wild type ETEC with Mabs and challenge the mice with the mixture as has been used for *V. cholerae* to demonstrate the prtective efficacy of antibodies to the TCP of *V. cholerae* (Sun et al, 1990). The results could be useful in defining the protective epitope(s) of CstH which is important in subunit vaccine design for ETEC.

9.3 Antigenic variation in CS3

The ability of a pathogen to vary the antigenic determinants is a mechanism by which it can evade the immune response of the host. In fimbriae and flagella variation occurs in the domains which are not involved in biogenesis and assembly. However, these domains have the potential to be replaced by foreign epitopes or accept the insertion of heterologous epitopes without disruption of the pili or flagella.

Some antigenic variations in CS3 had been suggested by Yolanda and Svennerholm (1990), who thought that the variation was dependent upon the biotype of the strain or co-expression of CS3 with CS2. Careful analysis of the variation in CS3 by sequencing and immunoblot analysis of the denatured and native CS3 from six different strains revealed that Mab 11:2 recognizes a conformational epitope which is unable to be detected by a Mab in one strain when the antigen is present in its native configuration. Mab 11:2 reacted with CS3 from five strains under both conditions and reacted only with denatured form of CstH in E248750-1 and in *E. coli* K-12 expressing CS3 of this strain from a recombinant *cst* operon. This strain differs from the others at amino acid #39 which is Lys instead of Asn. This phenomenon associated with epitope recognition has also been observed for CFA/I (Lopez-Vidal *et al.*, 1988) and for CS1, CS2 and CFA/I (Thomas *et al.*, 1985). The experiments presented in Chapter 4 clearly indicate that the variation is independent of the biotype of the strain or the co-expression of the CS3 with CS2. It seems likely that Mab 11:2 binds to a conformational epitope that includes amino acid #39 and that substitution of this residue affects antibody recognition. The epitope is shared by all six strains since they reacted with the Mab in a Western blot. One possible explanation is that the epitope is partially hidden within the pilus structure with an exposed part which includes amino acid #39. However, when this amino acid is substituted antibody binding to the pilus is abolished. However, a more feasible explanation is that the substitution of residue 39 simply inhibits binding of the MAb to its epitope because of both the positive charge and longer side chain on Lys compared to Asn. The simplest way to assess this could be by site-directed mutagenesis of residue #39. This sort of information could clearly be useful in studying the structure and assembly of CS3.

9.4 Epitope analysis of the *Cst*H

One approach to make a multivalent vaccine is the construction of a hybrid protein comprising different antigenic determinants. Expression of foreign epitopes in bacterial surface molecules has been used to elicit the production of antibodies against both vector and insert. Exposed regions of a protein that are most likely the epitopes and the variable regions of a pilin subunit are good candidate sites for insertion and expression of foreign epitopes. In addition, epitope analysis of a protein is also important in studies related to defining structure/function, the interactions during the immuneresponse and for the design of diagnostic reagents and vaccines. CstH is the major fimbrial subunit of CS3 and consequently epitope analysis is of importance in vaccine development against CS3 pili.

Since no variable regions suitable for the insertion of foreign epitopes could be identified, it was necessary to define the exposed areas or epitopes of this protein. Two epitopes were defined in initial experiments using two Mabs and C-terminal deleted CstH fusion proteins, but it proved impossible to define the precise sequence of the epitopes for these Mabs and for 19 Mabs produced in this study (Chapter 3) using C- and N-terminal deleted CstH fusion proteins. Several attempts such as changing culture conditions, method of sample preparation and experimental conditions failed to overcome the probable problems. Part of the reason for failure of fusion proteins to react with the Mabs may be due to the instability of the proteins.

Another reason for the failure of deletion mutagenesis in epitope mapping of the CstH may be due to existence of conformational epitopes in CstH as has been proposed for K88 (Van Zijderveld *et al.*, 1990). The importance of conformation in epitope recognition can be inferred from the effects of varying amino acid #39 on binding of Mab 11:2 (Chapter 4).

The characterization of the reactivity of the Mabs with hybrid CS3 pili (Chapter 8) showed that the Mabs could be grouped into different classes consistent with them being directed against different epitopes of CstH. The results were also consistent with the notion that the epitopes of CstH, at least for the Mabs used, are conformational. A group of Mabs were against an epitope including amino acids 99 and 100 (one insertion site) and some were against epitope(s) near the insertion sites amino acids 65-66 and 99-100 of mature CstH. Computer analysis of the secondary structure and surface accessibility predicted exposed regions containing these amino acids which is consistent with the recognized epitope consisting amino acids 99 and 100.

The results from epitope analysis and the antigenic variation study of CS3, in addition to the epitope analysis of K88 (Bakker *et al* 1992) may suggest that the epitopes of these pili are conformational. These data also suggest that random insertion mutagenesis or insertion of heterologous peptide into predicted area of the pilin subunit and amino acid substitution may be useful for epitope mapping, pilin-pilin interaction and receptor binding site analysis of CS3, and possibly other pili, as has also been successfully used for studying the folding of the LamB in the outer membrane (Charbit *et al.*, 1986, 1991) and characterizing the hypervariable domains of the major fimbrial subunit of P-fimbriae (van Die *et al.*, 1988).

9.5 Expression of heterologous peptides in CS3

The potential of CS3 pili as a delivery system for the expression of foreign epitopes on the surface of the bacteria has been investigated by insertion of two peptides in CstH, the major subunit of the CS3 pili. The flexibility of three areas for the insertion and expression of the foreign epitopes was examined. These areas were considered as exposed regions based upon computer programs for the prediction of the secondary

structure, associability and hydropathy of the proteins. Unique sites were introduced into these area by PCR mutagenesis. The epitopes which were chosen for insertion in CstH were the mature ST and amino acids 44-64 of hLT-B which are highly conserved in pLT-B and CT-B. These peptides have been used as models for simple ($LT\text{-}B_{44\text{-}64}$) and complex (ST) peptides. ST is a small, complex peptide with six cysteine residues which make 3 disulfide bonds that are necessary for its biological activity. Three epitopes have been demonstrated in this molecule (Takeda *et al.*, 1993).

The insertion of foreign epitopes between amino acids 108-109 was not successful suggesting that this is not a permissive site, possibly due to the toxicity of the hybrid protein for the cell. The insertion of the peptides between amino acids #65-66 and 99-100 of mature CstH resulted in four hybrid proteins, but expression from their natural promoter was poor and thus had to be expressed in the T7 promoter/RNA polymerase system to obtain sufficent protein for analysis. Since the regulatory regions have not been altered, the poor expression of the hybrid proteins presumably results from post transcriptional problems, which appear to only be reduced by increasing the overall level of transcription. For example, the stability of the hybrid mRNA may be adversely affected as a result of the insertion. This could be evaluated by northern analysis as well as pulse-chase studies to follow mRNA half-life. The use of coupled in vitro transcription/translation experiments may also facilitate studying the effect of the insertion of the epitopes on the stability of the *cstH* mRNA and its translation. Reconstitution of a *cst* operon in which the *cstH* hybrid genes replaced the wild type *cstH* has partially overcome the problems related to the expression of the hybrid proteins, however, the expression of CS3 was lower than in wild type ETEC and in clones containing the wild type *cst* operon. The improved level of expression of the CstH hybrid proteins from within a hybrid *cst* operon may be due to in part the additional

transcription when all of the CS3 biosynthesis genes are transcribed as a single transcript originating upstream from *cstA* (Jalajakumari, 1992). Transcription of the hybrid *cstH* as a part of a multicistronic mRNA may protect the hybrid *cstH* mRNA from degradation and reduce the translational problems.

Plasmid maintenance is also a problem which contributed to poor hybrid protein expression. This was seen when changing the selection marker of the vector from Ap to Cm. It is clear that the plasmids expressing the hybrid proteins are unstable and selection is required to maintain them, thus, Ap is unsatisfactory due to its degradation by the vector encoded β-lactamase. A more satisfactory solution for enhancing stability of the genetic constructs would be to incorporate the genes into the bacterial chromosome. For example, Hone *et al.* (1988) have developed a system based on the *hisOGD* gene. Alternatively, it may be that part of the problem is due to the high copy number of the vector, and single or low copy number vectors may be more suitable.

Besides reactivity with antibodies to CS3/CstH, the CstH::ST hybrid proteins also reacted with a Mab against ST and CstH::LT-B_{44-64} reacted with a polyclonal antiserum to CT but not with a Mab to LT-B. The Mab to LT-B was directed against the B pentamer, and so it is not surprising that it failed to detect the CstH::LT-B_{44-64}, however, recognition of these hybrid proteins by anti-CT indicate that the epitope is expressed and that would be it likely to be recognized by a polyclonal antiserum to LT-B. These results indicate that the epitopes inserted in CstH can be successfully expressed as the hybrid proteins and pili.

It was interesting to note however, that insertion of the foreign peptides affected the ability of some of the Mabs isolated here to recognize the hybrid pili. In particular, insertion between amino acids 99-100 and insertion of a more complex peptide (ST) had the greater affect on Mab binding to CstH. Thus, in spite of the potential of the CstH for

expression of foreign epitopes, there are some limitations on both insertion sites and the insert. Further investigations need to be performed to characterize other permissive sites in CstH and determine the types of peptide suitable for this delivery system. Perhaps, it may be more appropriate to define the X-ray crystallographic structure before embarking on extensive mutagenesis studies.

9.6 Immunogenicity of the hybrid CS3 pili

The immunogenicity of the hybrid CS3 pili was assessed by immunization of mice with *Salmonella typhimurium* strain G30 expressing these hybrids. This strain has been used for expression and immunization against K88 and K99 pili (Attridge *et al.*, 1988; Morona *et al.*, 1994; Stevenson and Manning, 1985). Plasmids encoding the hybrid proteins with insertion between amino acids 65-66 were transferred into *S. typhimurium* G30 and bacteria expressing hybrid pili were used for oral immunization of mice, which were boosted with formalin killed cells of the same bacteria. The sera and gut washing of the mice immunized with hybrid pili contained low levels of antibodies against CS3 compared to the titre of antibody produced against wild type pili. No antibodies against ST and LT-B could be detected. The failure of mice to respond to the hybrid proteins at the same level as the intact CS3 is probably a reflection of both poorer expression and the instability of the plasmids encoding hybrid CS3. CS3 fimbriae are known to be highly immunogenic (Reid *et al.*, 1993) which together with the instability of the plasmids may indicate that the plasmid has been lost in oral inocula and only antigen used in boosting led to the production of antibodies against CS3. It is also possible that in the hybrid protein the ST and LT-B epitopes are not presented as appropriate B-cell epitopes or processed into the relevant T-cell epitopes. ST is haptenic and becomes immunogenic when coupled with high molecular weight carriers and it may not become

immunogenic in CstH, although it can be in LT-B::ST hybrids (Cardenas and Clements, 1993). The failure of the production of antibodies against hybrid pili may be overcome by different immunization procedures or using purified hybrid pili to elicit the production of antibodies to both carrier and insert.

The toxicity of the hybrid pili has not been studied but it is necessary to define whether any of the hybrid proteins, especially CstH::ST hybrids, have any detrimental properties. Maintenance of any residual toxicity in the CstH::ST would pose a problem to the potential vaccine application of these constructs.

9.7 Concluding remarks

This study has sought to develop a new system, based on the CS3 subcomponent of the CFA/II of ETEC, for delivery of the foreign epitopes on the bacterial surface. In this regard, the structure of the CstH, the major subunit of the CS3, was analysed, both by computer analysis of the amino acid sequence of the protein and by experimental study of its antigenic variation together with epitope mapping using a panel of Mabs generated in this study. CstH is highly conserved among the strains studied. Epitope analysis led to the suggestion that the Mabs are against different epitopes which are conformational. Computer analysis of the secondary structure of the CstH defined exposed area of CstH which can tolerate the insertion of the heterologous peptide. Experimental insertion of foreign peptides (a epitope of LT-B and ST) into two regions of CstH confirmed this assumption and hybrid proteins were expressed from both the bacteriophage T7 promoter and natural *cst* operon promoters. More interestingly, the hybrid proteins are able to be assembled as pili on the surface of the bacteria harbouring plasmids encoding hybrid proteins. The CstH::ST hybrid proteins could also be recognized by a Mab directed against ST and the hybrid proteins containing LT-B

epitope were reacted with antiserum against CT. The most interesting feature of this system is that, the CstH can accept the insertion of a complex peptide (ST) with cys residues in contrast to other delivery systems which are sensitive to insertion of complex foreign epitopes. This may be due to the composition of the CstH which has no cys residues of its own that can interfere with those of the foreign epitope. This feature may give CS3 an advantage for the delivery of complex epitopes which cannot be expressed on other carriers.

Although the hybrid proteins were expressed and reacted with antibodies to CS3 and foreign epitopes, there are still some limitations in regard to the insert, insertion sites and stability of the plasmids leading to poor expression of the hybrid proteins. The more complex insert has a greater effect on the structure of CstH but this carrier is still more flexible than others. These limitations should be investigated and overcome in the future to find the most stable state of the hybrid genes leading to the high level expression of hybrid proteins with the minimum toxicity that can be assembled as pili.

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