The EMBO Journal vol.8 no.11 pp.3535-3540, 1989

Cloning and expression in *Escherichia coli* of *Haemophilus influenzae* fimbrial genes establishes adherence to oropharyngeal epithelial cells

S.Marieke van Ham, Frits R.Mooi[1], M.Gita Sindhunata, Wil R.Maris and Loek van Alphen

Department of Medical Microbiology, University of Amsterdam, Meibergdreef 15, NL-1105 AZ Amsterdam and [1]Department of Molecular Microbiology, National Institute of Public Health and Environmental Protection, PO Box 1, NL-3720 BA Bilthoven, The Netherlands

Communicated by P.H.Mäkelä

In this report the first example of functional expression of a fimbrial gene cluster of a non-enteric human pathogen in *Escherichia coli* is described. This is shown for *Haemophilus influenzae* fimbriae which mediate adherence to oropharyngeal epithelial cells. A genomic library of *H.influenzae* type b, strain 770235f$^+$b^o, was constructed using a cosmid vector and screened with a synthetic oligonucleotide probe derived from the N-terminal sequence of the fimbrial subunit of *H.influenzae*. Four cosmid clones were found which hybridized to this oligonucleotide probe. *Escherichia coli* strains harbouring these clones expressed the *H.influenzae* fimbriae at their cell surface, as was demonstrated in a whole-cell ELISA and by immunogold electron microscopy using a monoclonal antibody specific for the *H.influenzae* fimbriae. Surface expression could be maintained during subcloning until a minimal *H.influenzae* DNA insert of ~8.1 kb was obtained. *Escherichia coli* strains harbouring the 8.1 kb *H. influenzae* DNA were able to cause a mannose-resistant adherence to oropharyngeal epithelial cells and a mannose-resistant haemagglutination of human AnWj-positive erythrocytes. The nucleotide sequence of *hifA*, the gene encoding the major fimbrial subunit, was determined. The predicted amino acid sequence shows a significant homology with a number of *E.coli* fimbrial subunits.

Key words: adherence/fimbriae expression/*Haemophilus influenzae*/pathogenesis/sequence

Introduction

The Gram-negative bacterium *Haemophilus influenzae* is an important human pathogen, causing serious systemic infections in young children. These infections include meningitis and also mucous membrane infections, such as chronic bronchitis (Turk, 1985). Adherence is generally believed to be the first step in the pathogenesis of many infectious diseases and probably represents a common step in both systemic and mucosal *H.influenzae* infections. Therefore, more knowledge about the components mediating this adherence may result in a vaccine that is effective against both systemic and mucosal *H.influenzae* infections. For many pathogenic bacteria it has been shown that adherence is mediated by fimbriae (Pearce and Buchanan, 1980). The genetic organization of various *Escherichia coli* fimbriae has already been examined in detail and the expression of particular gene products at the cell surface could be correlated to the ability of *E.coli* to adhere to host cells (Lindberg *et al.*, 1986; Hanson and Brinton, 1988). However, expression of fimbriae of non-enteric human pathogens at the cell surface of *E.coli* has not yet been reported. The adherence of *H.influenzae* to various cell types is also mediated by fimbriae. Fimbriated forms of *H. influenzae* adhere better to human nasopharyngeal epithelial cells and human oropharyngeal cells than non-fimbriated forms (Guerina *et al.*, 1982; Pichichero *et al.*, 1982). This is particularly of interest, since the nasopharynx is generally assumed to act as the port of entry for *H. influenzae*. Fimbriated bacteria also cause a mannose-resistant agglutination of human erythrocytes (MRHA), which express the blood group AnWj antigen (van Alphen *et al.*, 1986; Poole and van Alphen, 1988). The adherence to oropharyngeal cells and MRHA can be inhibited by monoclonal antibodies directed against the fimbriae (van Alphen *et al.*, 1988). Moreover, isolated fimbriae bind to oropharyngeal epithelial cells and cause MRHA (van Alphen *et al.*, 1988).

In this paper we describe the cloning in *E.coli* of the structural genes involved in the biosynthesis of the fimbriae of *H.influenzae* type b. We show that the cloned *H.influenzae* DNA fragment confers upon *E.coli* the ability to produce morphologically and functionally intact fimbriae. This will allow us to study in *E.coli* the role of *H.influenzae* fimbriae in the pathogenesis of both systemic disease and respiratory tract infections, without interference of other *H.influenzae* virulence products. The nucleotide sequence of the structural gene encoding the major fimbrial subunit, *hifA*, is presented and the significant homology between *hifA* and the major subunits of the *E.coli* type 1 and Pap fimbriae is discussed.

Results

Identification of the H.influenzae fimbrial gene (hifA) with an oligonucleotide probe

In order to identify clones containing the fimbrial genes of *H.influenzae* strain 770235f$^+$b^o, we synthesized an oligonucleotide probe derived from the N-terminal amino acid sequence of the 23 kd fimbrial subunit of the type b strain AO2 (Guerina *et al.*, 1985) (Figure 1). This sequence showed complete identity to the first 16 amino acid residues of the fimbrial subunit derived from strain 770235f$^+$b^o, except for the first residue (data not shown), and was therefore presumed to be representative for the 770235f$^+$b^o subunit. The oligonucleotide was derived from residues 12-22 because this region contained predominantly amino acids which show limited codon degeneracy. Codon usage

```
          12  13  14  15  16  17  18  19  20  21  22
protein   Phe-Phe-Gly-Lys-Val-Val-Glu-Asn-Thr-Cys-Lys
oligo  5' TTT-TTT-GGT-AAA-GTT-GTT-GAA-AAT-ACA-TGT-AAA 3'
```

Fig. 1. Synthetic oligonucleotide probe derived from the N-terminal amino acid sequence of the *H.influenzae* fimbrial subunit of strain AO2. The nucleotides at the wobble positions of the codons were chosen on the basis of codon usage data of *H.haemolyticus* (Caserta *et al.*, 1987). The corresponding amino acid sequence of residues 12–22 of the fimbrial subunit is also shown.

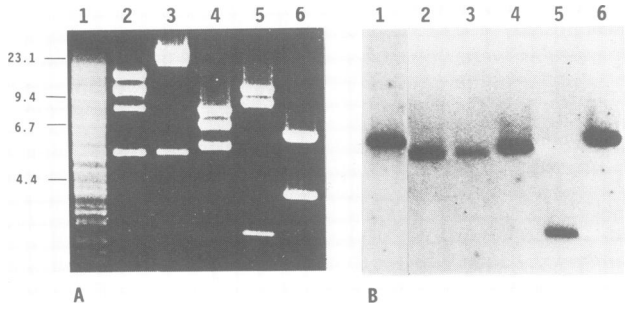

Fig. 2. Restriction enzyme analysis of *H.influenzae* DNA containing the *hifA* gene. (**A**) DNA was digested with *Pst*I and separated on a 0.7% agarose gel. (**B**) After gel-electrophoresis, the DNA was transferred to a nylon membrane, and hybridized to the labelled oligonucleotide probe from Figure 1. **Lanes: 1**, chromosomal DNA derived from *H.influenzae* strain 770235f$^+$b^o; **2**, cosmid pMH100; **3**, cosmid pMH200; **4**, cosmid pMH300; **5**, cosmid pMH400; **6**, phasmid pMH001. The numbers on the left indicate the sizes (in kilobase pairs) of marker DNA fragments.

data were obtained from the *Hha*I methyl transferase gene of *Haemophilus haemolyticus* (Caserta *et al.*, 1987).

The oligonucleotide probe did not hybridize to genomic restriction digests of *E.coli* strain LE392 or DH5α under the experimental conditions used. However, hybridization with *Pst*I-digested chromosomal DNA from strain 770235f$^+$b^o revealed a single fragment (6.0 kb) (Figure 2, lane 1), which suggested the presence of a single fimbrial subunit gene (designated *hifA*).

Molecular cloning of the 770235f$^+$b^o hifA gene

The 6.0 kb *Pst*I fragment was isolated and cloned into pEMBL8. One of the plasmids harbouring the 6.0 kb *Pst*I fragment was designated pMH001. *Escherichia coli* strains harbouring pMH001 did not produce *H.influenzae* fimbriae, as determined by immunogold electron microscopy and a whole-cell ELISA (see below). This suggested that the 6.0 kb *Pst*I fragment does not contain all the information necessary for fimbriae expression, or that *E.coli* is unable to produce functionally intact *H.influenzae* fimbriae.

To see if it was possible to produce functionally intact *H.influenzae* fimbriae in *E.coli*, larger DNA fragments were cloned using a cosmid vector. A genomic library consisting of 1056 recombinant clones was constructed by cloning partially *Sau*3A digested chromosomal DNA of the *H. influenzae* strain 770235f$^+$b^o into the cosmid pHC79 followed by transduction into *E.coli* strain LE392. The library was screened with the synthetic oligonucleotide derived from the fimbrial subunit (Figure 1). Four clones showed a strong positive hybridization signal and were found to produce the *H.influenzae* fimbriae at their cell surface (see below). The cosmids contained in these clones were

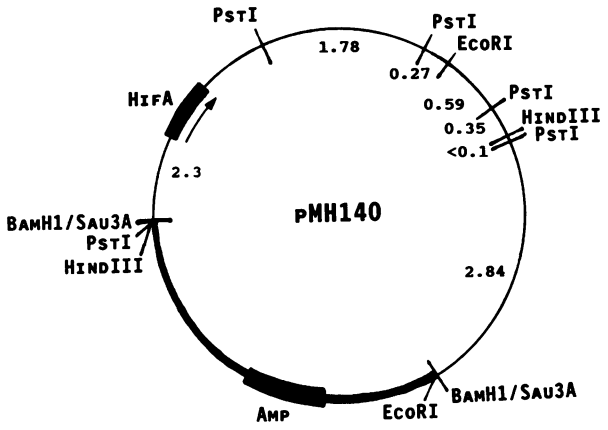

Fig. 3. Restriction enzyme map of the 8.13 kb insert in pMH140 that encodes the *H.influenzae* fimbriae. pEMBL8 DNA is indicated by the thick line. The position and the orientation of the fimbrial subunit gene, *hifA*, are indicated. Distances between restriction sites are given in kilobases. Amp refers to the ampicillin-resistance gene of pEMBL8.

designated pMH100, pMH200, pMH300 and pMH400 and contained *H.influenzae* DNA varying in size from 18.6 to 48.2 kb. Cosmid DNA was purified, digested with *Pst*I and analysed by Southern hybridization using the synthetic oligonucleotide as a probe (Figure 2). All cosmid clones contained a single *Pst*I fragment hybridizing to the probe. The size of the hybridizing fragments was similar for clones pMH100 and pMH200 (5.2 kb), but differed from the corresponding fragments (5.6 and 3.1 kb) of the other clones (pMH300 and pMH400). The hybridizing fragment of each clone was smaller than the corresponding fragment of clone pMH001, which contains the *Pst*I fragment detected in genomic blots of strain 770235f$^+$b^o. The finding that the size of the *Pst*I fragment which contains the *hifA* gene differs between the various clones indicates that this fragment is not an internal fragment in the *H.influenzae* DNA contained in the cosmids, but a flanking fragment.

Expression of the H.influenzae fimbriae in E.coli

Escherichia coli strains with the four cosmids harbouring the *hifA* gene were analysed for their ability to confer upon *E.coli* the ability to produce *H.influenzae* fimbriae at its cell surface. To this end, the *E.coli* strains were tested in a whole-cell ELISA for reactivity with MoAb 6HE8, which is specific for the *H.influenzae* fimbriae (van Alphen *et al.*, 1988) (Table I, column 2). All four strains reacted with MoAb 6HE8, indicating that the fimbrial epitope that is recognized by this MoAb was produced, transported and assembled into its native conformation at the cell surface of *E.coli*. The reactivity with MoAb 6HE8 varied between the various clones and was not as strong as that of *H.influenzae* strain 770235f$^+$b^o.

Subcloning of the H.influenzae DNA harbouring hifA

In order to restrict the size of the cloned *H.influenzae* DNA to the minimal size necessary for fimbriae expression, subcloning was performed. Clone pMH100, which contains a *H.influenzae* DNA insert of 35.7 kb, was partially digested with *Sau*3A to obtain fragments with sizes between 5 and 10 kb. After isolation with porous glass, these fragments were subcloned into the unique *Bam*HI restriction site of the phasmid pEMBL8 and the mixture was used to transform

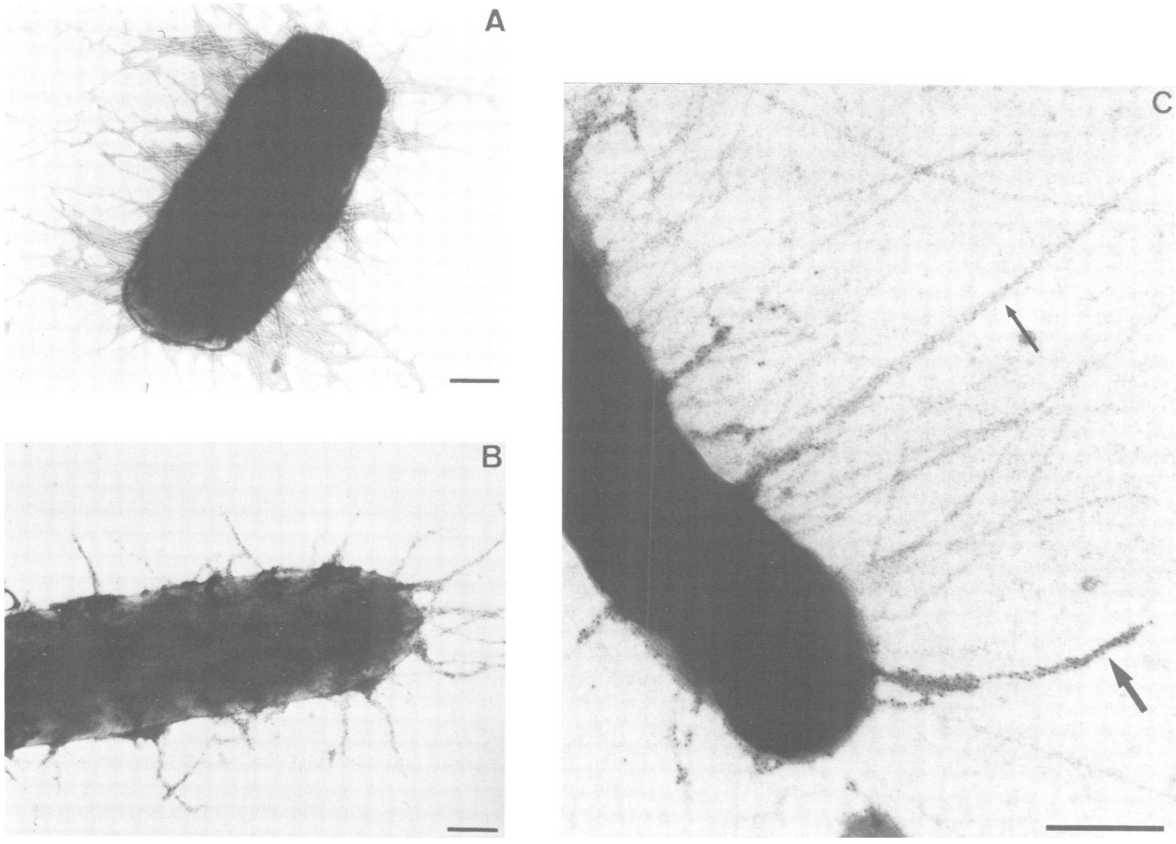

Fig. 4. Electron micrographs of immunogold labelled DH5α derivatives. Bacteria were incubated with MoAb 6HE8 followed by gold-conjugated Protein A. Subsequently the bacteria were counterstained with phosphotungstic acid. (**A**) *Escherichia coli* clone DH5α/pMH001; (**B**,**C**) *E.coli* DH5α/pMH140. Bars, 0.3 μm. The thick arrow in (C) indicates the *H.influenzae* fimbriae and the thin arrow indicates the type 1 *E.coli* fimbriae.

E.coli strain DH5α. A number of positive transformants were found after screening with MoAb 6HE8. The smallest *H.influenzae* DNA fragment that still conferred the ability to produce fimbriae was contained in pMH140, which had a DNA insert of 8.1 kb. Restriction enzyme analysis of pMH140 showed that several restriction enzymes had recognition sites in the cloned DNA fragment (Figure 3).

Immunogold electron microscopy

In order to examine the expression of the *H.influenzae* fimbrial gene cluster in *E.coli* in more detail, immunogold electron microscopy with MoAb 6HE8 was performed on various clones and subclones. *Escherichia coli* strains containing *H.influenzae* DNA that did not hybridize to the oligonucleotide probe were not labelled with MoAb 6HE8 (results not shown). Furthermore, *E.coli* strains harbouring plasmid pMH001 also were not labelled (Figure 4A). Gold spheres were not seen on the bacterial cell surface nor on the type 1 fimbriae of the *E.coli* strain. *Escherichia coli* strains harbouring plasmid pMH140 expressed hair-like fimbriae, which resembled *H.influenzae* fimbriae (Figure 4B). These haemophilus-like fimbriae were labelled with gold spheres along the axis of the fimbriae, in contrast to the morphologically different type 1 fimbriae of these *E.coli* strains. Sparse labelling of the bacterial cell surface was presumably due to folding of the *H.influenzae* fimbriae over the cell surface. Expression of these fimbriae did not seem to affect the expression of the *E.coli* type 1 fimbriae, since one single bacterium could express both *H.influenzae* fimbriae and type 1 fimbriae (Figure 4C).

Mannose-resistant adherence and haemagglutination

Various *E.coli* strains carrying recombinant cosmids and plasmids were tested for *H.influenzae*-like adherence to oropharyngeal epithelial cells. The assay was performed in the presence of mannose to abolish unwanted adherence phenomena due to the *E.coli* type 1 fimbriae. Adherence to oropharyngeal cells was in all cases correlated to the presence of *H.influenzae* fimbriae on the bacterial cell surface, and *E.coli* clones not expressing these fimbriae did not adhere (Table I, column 3).

The ability to cause a MRHA of only AnWj-positive erythrocytes was in the same way correlated to the expression of the *H.influenzae* fimbriae on the *E.coli* cell surface (Table I, columns 4 and 5). The amount of adherence and MRHA paralleled the amount of fimbriae on the bacterial cells.

Nucleotide sequence of the hifA gene

The oligonucleotide probe derived from the N terminus of the fimbrial subunit was used to map the *hifA* gene within pMH140. It appeared that the gene was contained within a 2.3 kb *Pst*I DNA fragment. This fragment was subcloned into pEMBL8 and the resulting clone pMH142 was used to sequence ~1 kb of this fragment on both strands. Part of the nucleotide sequence is shown in Figure 5. An open reading frame was identified between bases 115 and 756, coding for a polypeptide of which a part was identical to the N-terminal amino acid sequences of the AO2 and 770235f^{+}b^{o} fimbrial subunits. Thus we conclude that this open reading frame represents the *hifA* gene. From the DNA sequence it can be inferred that the fimbrial subunit is

Table I. Adherence *in vitro* of bacteria to human oropharyngeal cells and erythrocytes

Strain	ELISA titre with MoAb 6HE8[a]	Epithelial cell adherence[b]	MRHA[c]	
			AnWj-positive erythrocytes	AnWj-negative erythrocytes
770235f$^+$b^o	1:512	+++	++	−
770235f^{o}b^o	<1	−	−	−
LE392/pMH100	1:64	+	+	−
LE392/pMH300	1:32	++	+	−
LE392/pMH400	1:32	+	+	−
DH5α/pMH140	1:64	++	+	−
DH5α/pMH001	<1	−	−	−

[a]Relative expression of *H.influenzae* fimbriae was measured by reactivity with MoAb 6HE8 in a whole-cell ELISA with 2-fold dilutions of the bacteria.
[b]Adherence was expressed as +++ if >200 bacteria bound per epithelial cell, as ++ for 50−200 bacteria, as + for 5−50 bacteria and as − for <5 bacteria.
[c]Mannose-resistant haemagglutination was expressed as ++ for strongly positive agglutination, as + for positive and as − for negative.

```
                                                                    TTTTTTGGAAAACAAATCTTGCTGTTTATTAAGGCTTTAGCATTTTAATAAACGG   55
                                                                                                        -35

TTTATATTAATCTTTTGCCTTATTTATAAGGCACAAACCTCTTTATGGAGCAATTTATT ATG AAA AAA ACA CTT CTT GGT AGC TTA  141
   -10                                                 RBS              Met Lys Lys Thr Leu Leu Gly Ser Leu

ATT TTA TTG GCA TTT GCG GGA AAT GTG CAG GCG GAT ATT AAT ACT GAA ACA TCT GGT AAA GTT ACT TTT TTT  213
Ile Leu Leu Ala Phe Ala Gly Asn Val Gln Ala Asp Ile Asn Thr Glu Thr Ser Gly Lys Val Thr Phe Phe
                                                 ↑

GGT AAG GTT GTT GAG AAT ACT TGT AAA GTG AAA ACA GAG CAT AAA AAT CTG AGT GTA GTA TTA AAT GAT GTG  285
Gly Lys Val Val Glu Asn Thr Cys Lys Val Lys Thr Glu His Lys Asn Leu Ser Val Val Leu Asn Asp Val

GGT AAA AAT AGC TTA TCT ACT AAA GTA AAT ACC GCA ATG CCA ACG CCA TTT ACG ATT ACA TTA CAA AAT TGT  357
Gly Lys Asn Ser Leu Ser Thr Lys Val Asn Thr Ala Met Pro Thr Pro Phe Thr Ile Thr Leu Gln Asn Cys

GAC CCA ACT ACG GCA AAT GGT ACT GCA AAT AAA GCT AAT AAA GTT GGG CTA TAT TTC TAT TCT TGG AAA AAT  429
Asp Pro Thr Thr Ala Asn Gly Thr Ala Asn Lys Ala Asn Lys Val Gly Leu Tyr Phe Tyr Ser Trp Lys Asn

GTA GAT AAA GAA AAT AAC TTT ACG TTG AAA AAT GAA CAA ACT ACG GCA GAT TAC GCA ACT AAT GTT AAT ATT  501
Val Asp Lys Glu Asn Asn Phe Thr Leu Lys Asn Glu Gln Thr Thr Ala Asp Tyr Ala Thr Asn Val Asn Ile

CAA CTT ATG GAA AGT AAT GGT ACA AAG GCG ATT TCA GTA GTG GGT AAA GAA ACG GAA GAT TTT ATG CAT ACG  573
Gln Leu Met Glu Ser Asn Gly Thr Lys Ala Ile Ser Val Val Gly Lys Glu Thr Glu Asp Phe Met His Thr

AAT AAT AAT GGA GTA GCA TTA AAC CAA ACC CAC CCA AAT AAT GCT CAT ATT TCA GGA AGT ACA CAA TTA ACC  645
Asn Asn Asn Gly Val Ala Leu Asn Gln Thr His Pro Asn Asn Ala His Ile Ser Gly Ser Thr Gln Leu Thr

ACT GGT ACT AAT GAA CTT CCT CTC CAC TTT ATC GCC CAA TAT TAC GCA ACA AAT AAA GCA ACT GCT GGC AAA  717
Thr Gly Thr Asn Glu Leu Pro Leu His Phe Ile Ala Gln Tyr Tyr Ala Thr Asn Lys Ala Thr Ala Gly Lys

GTA CAA TCC TCA GTT GAT TTC CAA ATT GCT TAC GAA TAA TTCCTAATGTAATGCAAGAGAAACTAAGCCCACGTGTTTATTA  799
Val Gln Ser Ser Val Asp Phe Gln Ile Ala Tyr Glu ---                                  ←

TATCCCGCGTGGGCTTACGCCCACCCTACAAC  831
←      →      ←
```

Fig. 5. Nucleotide sequence of the gene encoding the major fimbrial subunit of strain 770235f$^+$b^o (*hifA*). The predicted amino acid sequence is shown below the nucleotide sequence. The putative promoter sequences (−35 and −10) and the ribosome binding site (RBS) are shown, as well as two putative rho-dependent terminators. The vertical arrow indicates the cleavage site for signal peptidase.

synthesized as a precursor containing a typical signal peptide of 20 amino acids and a mature protein of 194 amino acids. The predicted mol. wt of the processed protein is 21.09 kd, which is in good agreement with the apparent mol. wt of 23 kd in SDS−PAGE gels. There are putative −35 and −10 promoter sequences, which are separated by 16 bases. Both sequence and separation are close to the consensus for these sequences in *E.coli* (Harley and Reynolds, 1987). There are two potential initiation codons, located at positions 100 and 115. Initiation is probably initiated more effectively from the second initiation codon, because it is preceded by a region showing the largest degree of homology with the Shine−Dalgarno sequence (Gold and Stormo, 1987). Downstream of the translational termination codon two inverted repeats are found that may act as a rho-dependent transcriptional terminators (von Hippel *et al.*, 1984). The first inverted repeat is the most likely terminator, since its predicted stem−loop structure in the mRNA transcript is the most stable one. The predicted amino acid sequence of the *hifA* gene product was compared with sequences stored in NBRF Protein Sequence Database, updated to September 1987, by using FASTP software (Pearson and Lipman, 1988). The best match was to *focA*, the major fimbrial subunit of the type IC fimbriae (van Die *et al.*, 1984). There was 27.8%

identity over 194 amino acids. Other high-scoring matches were *fimA* of type 1A fimbriae of *E.coli* (Klemm, 1984) (22.1% identity over 194 amino acids), *papA* of P-pili of the uropathogenic *E.coli* strain J96 (Båga *et al.*, 1984) (19.4% identity over 194 amino acids) and *fim2* of the fimbriae of *Bordetella pertussis* (Livey *et al.*, 1987) (19.2% identity over 194 amino acids).

Discussion

We have cloned the *hifA* gene of *H.influenzae* strain 770235f$^+$b^o into *E.coli* and established that *E.coli* is able to express the *H.influenzae* fimbriae at its cell surface. Several observations indicate that *hifA* is located within a gene cluster involved in biosynthesis of the *H.influenzae* fimbriae. A 6.0 kb *Pst*I fragment which contained a complete copy of the *hifA* gene was unable to confer the ability to produce fimbriae upon *E.coli*, suggesting that additional genes were required for this. This assumption was confirmed when DNA fragments that contained a larger part of the *H.influenzae* genome and overlapped with the *Pst*I fragment were found to confer the ability to produce fimbriae. After partial digestion with *Sau*3A, the smallest size of *H.influenzae* DNA which appeared to be required for fimbriae production was 8.1 kb. Smaller DNA fragments, although harbouring a complete copy of the *hifA* gene, were unable to confer the ability to produce fimbriae upon *E.coli* (results not shown). Thus we presume that the *H.influenzae* fimbrial gene cluster encompasses ~8 kb, which is similar to the size of various fimbrial gene clusters of *E.coli*.

A number of different approaches were used to characterize the *H.influenzae* fimbriae produced by *E.coli*. With a whole-cell ELISA using a monoclonal antibody directed against the *H.influenzae* fimbriae, it could be demonstrated that *E.coli* strains harbouring the *H.influenzae* gene cluster are able to transport the *hifA* product to the cell surface. Using immunogold electron microscopy, we could show that the *H.influenzae* fimbriae produced by *E.coli* were morphologically identical to the fimbriae produced by *H. influenzae*. Finally, the *H.influenzae* fimbriae produced by *E.coli* were functional, as they were able to mediate adherence to oropharyngeal epithelial cells and mannose-resistant haemagglutination of human AnWj-positive erythrocytes. From these data, we conclude that *E.coli* is able to perform all steps required for the biogenesis of *H.influenzae* fimbriae and that the cloned fimbrial gene cluster contains the gene encoding the adhesin which mediates both binding to oropharyngeal cells and binding to erythrocytes. As far as we know, this is the first description of the expression of fimbriae of a non-enteric pathogen at the cell surface of *E.coli*.

So far, only one type of fimbrial subunit has been purified from a *H.influenzae* type b isolate (Guerina *et al.*, 1985). This subunit is encoded by only one gene copy on the bacterial chromosome, since Southern hybridization of the synthetic oligonucleotide derived from its N-terminal amino acid sequence to *Pst*I-digested chromosomal DNA resulted in a single hybridizing fragment.

A 2.3 kb *Pst*I DNA fragment harbouring the *hifA* gene was sequenced for ~1 kb on both strands. It appeared that *hifA* codes for a protein with a typical signal peptide. The N terminus of the mature *hifA* product could be identified by comparison with the N-terminal amino acid sequence of *H.influenzae* fimbrial subunits as determined by us and others (Guerina *et al.*, 1985). The first 16 amino acids of the predicted *hifA* protein sequence were identical to those that were determined for the subunit of this strain. Between the *hifA* subunit and the N-terminal sequence of the fimbrial subunit derived from strain AO2 (Guerina *et al.*, 1984) two differences are observed at positions 1 and 37. The difference at the first position was already expected, since this had already been noticed after comparison of the N-terminal protein sequence of both strains. At position 37 the AO2 fimbrial subunit contains the residue threonine instead of valine, which is found for the *hifA* subunit. Comparison of the predicted *hifA* protein sequence with the NBRF database reveals a highly significant homology to the major fimbrial subunits of type 1C (van Die *et al.*, 1984), type 1A (Klemm, 1984) and P-fimbriae (Båga *et al.*, 1984) of *E.coli*. This finding suggests that the *H.influenzae* fimbriae are closely related to *E.coli* fimbriae. Moreover, the predicted amino acid sequence contains two cysteines in the N-terminal half of the protein and a penultimate tyrosine, which are also typical characteristics of *E.coli* fimbrial subunits. At the moment it is not known whether the major fimbrial subunit is the fimbrial adhesin itself or whether the adhesin is encoded by a gene distinct from *hifA*.

Several reports indicate the existence of a second adhesin distinct from the fimbrial adhesin (Sable *et al.*, 1985; Loeb *et al.*, 1988). Non-fimbriated *H.influenzae* strains adhere better than fimbriated variants to the tissue culture cell line HEp-2 (Sable *et al.*, 1985) and fimbriated and non-fimbriated forms do not compete for the same receptor sites on adenoidal tissue in two adherence assays (Loeb *et al.*, 1988). The characterization of this second adhesin may be facilitated by mutants which are unable to express fimbriae. The successful cloning of the *H.influenzae* fimbrial gene cluster will allow the construction of such well-defined mutants as well as the analysis of individual fimbrial constituents in *H.influenzae* and in *E.coli*. Moreover, by using *E.coli* strains expressing *H.influenzae* fimbriae we are now able to study their role in the pathogenesis of the both systemic and mucosal *H.influenzae* infections at the molecular level, without interference of other *H.influenzae* virulence factors.

Materials and methods

Bacterial strains, DNA cloning vectors and growth conditions

Haemophilus influenzae type b strain 770235 (outer membrane protein subtype 2, lipopolysaccharide serotype 2 and biotype I) was isolated from the cerebrospinal fluid of a patient with meningitis. The heavily fimbriated, acapsular variant of this strain, 770235f$^+$b^o, was selected as described before (van Alphen *et al.*, 1988). *Haemophilus influenzae* was grown at 37°C with shaking (120 r.p.m.) in 1 l of brain heart infusion broth supplemented with X and V factors (10 mg of each per litre).

The cosmid vector pHC79 (Hohn and Collins, 1980) was used to construct a 770235f$^+$b^o genomic library. Plasmid subcloning was accomplished using pEMBL8 (Dente *et al.*, 1983).

The *E.coli* K-12 strain LE392 [F$^-$, *hsdR514* (r_k^-,m_k^-), *supE44, supF58, lacY1, galK2, galT22, metB1, trpR55*$^-$] (Maniatis *et al.*, 1982) was used as the host for the cosmid vector pHC79 and *E.coli* K-12 strain DH5α [F$^-$, *endA1, hsdR17* (r_k^-,m_k^-), *supE44, thi1, Lda*$^-$, *recA1, gyrA96, relA1*] (Gibco-BRL, Gaithersburg, USA) was used for transformation experiments with derivatives of phasmid pEMBL8. The *E.coli* strains were grown at 37°C, either in NZYM medium (Maniatis *et al.*, 1982) with shaking (120 r.p.m.) or on NZYM agar plates. Ampicillin (100 μg/ml; Sigma Chemical Co, St Louis, MO) was added where appropriate.

Purification of fimbriae and N-terminal amino acid determination

Fimbriae of *H.influenzae* strain 770235f$^+$b^o were purified as described by van Alphen *et al.* (1988). The first 16 residues of the N-terminal amino acid sequence of the 23 kd fimbrial subunit were analysed with a gas-phase amino acid sequenator. The instrument used was an Applied Biosystems Model 470A Protein Sequencer, equipped on-line with a Model 120A PTH Analyzer.

DNA techniques

Haemophilus influenzae chromosomal DNA was prepared as described by van Embden *et al.* (1983). Southern blotting of restriction endonuclease-digested DNA was performed as previously described (Reed and Mann, 1985). Hybridization was performed overnight at 30°C in a solution of 40 mM Tris (pH 7.5), 1 M NaCl, 1% SDS, 10 × Denhardt's solution and 50 μg/ml herring sperm DNA. The membranes were washed with 5 × SSPE containing 1% SDS at 47°C.

The 6.0 kb *Pst*I fragment harbouring the *hifA* gene was cloned by digesting chromosomal DNA with *Pst*I and separating the DNA fragments by agarose gel-electrophoresis. *Pst*I fragments with molecular sizes of ~6.0 kb were isolated with porous glass (Geneclean, BIO 101, La Jolla, CA) and inserted in pEMBL8 cleaved with *Pst*I. After transformation, colonies harbouring the *hifA* gene were identified by colony-blotting, using the labelled synthetic oligonucleotide as a probe. Standard procedures were used for the preparation of plasmid DNA, cleavage, linkage, transformation into *E.coli* and packaging DNA (Maniatis *et al.*, 1982). All enzymes were obtained from either Boehringer GmbH (Mannheim, FRG) or Promega (Madison, WI) and were used according to the instructions provided by the manufacturer.

Construction and screening of 770235f$^+$b^o genomic library

Total *H.influenzae* DNA was partially cleaved by *Sau*3A until fragments with predominant sizes between 35 and 45 kb were obtained. Cosmid pHC79 DNA was digested with *Bam*HI, treated with calf intestine alkaline phosphatase and ligated to the *Sau*3A-digested DNA. After *in vitro* packaging and transduction into the *E.coli* strain LE392, ~3000 independent ampicillin-resistant clones were obtained.

Colony hybridization with a synthetic oligonucleotide probe derived from the *H.influenzae* fimbrial subunit was used to screen 1056 colonies of the 770235f$^+$b^o genomic library as follows. After growth on NZYM-agar containing 100 μg/ml ampicillin, the bacterial colonies were transferred to dry Gene Screen-Plus membranes (Du Pont, Boston, MA). The membranes were then put, colony side up, on several sheets of Whatmann 3MM paper saturated with 0.5 M NaOH. This was placed for 5 min in a water bath with boiling water just above the water level, resulting in colony lysis. The membranes were neutralized in 1 M Tris−HCl (pH 7.5) and bacterial debris was thoroughly rubbed off.

The oligonucleotide probe was labelled with [γ-^{32}P]dATP (Amersham Corp., UK) using T4 polynucleotide kinase (Pharmacia Fine Chemicals, Piscataway, NJ) (Maniatis *et al.*, 1982) and hybridization was performed as described above.

DNA sequence analysis

DNA sequence analysis was performed on both strands of the fimbrial subunit gene by the dideoxy nucleotide chain termination method with the vector pEMBL8 (Sanger *et al.*, 1977). For optimal results T7 DNA polymerase (Pharmacia) was used in combination with the Sequenase kit of US Biochemicals (Cleveland, OH). [α-^{35}S]dATP was purchased from Amersham.

Whole-cell ELISA

The relative reactivity of various (sub)clones with MoAb 6EH8 was determined in a whole-cell ELISA, as previously described (Abdillahi and Poolman, 1987). The following adjustments were made. After coating of the microtitre plates with whole bacteria, the plates were washed with phosphate-buffered saline (PBS) (pH 7.4) containing 0.05% Tween 20. The MoAb 6HE8, previously described by van Alphen *et al.* (1988), was diluted 1:100 in PBS containing 0.3% bovine serum albumin (BSA) and 0.1% NaN_3 and the Protein A−peroxidase conjugate (Sigma) was diluted to a final concentration of 0.2 μg/ml in PBS with 0.3% BSA and 0.1% Tween 80.

Immunogold electron microscopy

Bacteria (1.5 × 10^7/ml) were incubated with MoAb 6HE8 in a 1:10 dilution in PBS with 0.5% BSA for 45 min at 37°C. After washing twice with PBS, the bacteria were incubated for 45 min at 37°C with gold-labelled Protein A (which was kindly provided by J.Weel). The gold-probe was prepared as previously described (Slot and Geuze, 1985) and used with an optical density at 520 nm of 0.06. After two more washings the bacteria were applied to carbon-coated copper grids. The grids were stained with 2% phosphotungstic acid and examined in a Philips EM 300 electron microscope at 60 kV.

Mannose-resistant adherence of H.influenzae to oropharyngeal epithelial cells and MRHA

Adherence of *H.influenzae* to oropharyngeal epithelial cells was determined as described previously (van Alphen *et al.*, 1987). The assay was performed in the presence of 0.5% mannose to abolish adherence mediated by type 1 fimbriae. MRHA was examined by adding mannose to a final concentration of 0.5% in a standard tube blood-grouping assay which was also described before (van Alphen *et al.*, 1986).

Acknowledgements

We thank H.G.J.van der Heide and H.M.Hodemaekers for synthesizing the oligonucleotide probes, J.Weel for immunological reagents and R.Amons and J.de Graaf for amino acid analyses. Amino acid sequences were determined at the Gas Phase Sequenator Facility (Department of Medical Biochemistry, State University of Leiden, The Netherlands), which is supported by the Netherlands Organization for the Advancement of Pure Research (ZWO) through the Netherlands Foundation for Chemical Research (SON).

References

Abdillahi,H. and Poolman,J.T. (1987) *FEMS Microbiol. Lett.*, **48**, 367−371.
Båga,M., Normark,S., Hardy,J., O'Hanley,P., Lark,D., Olsson,O., Schoolnik,G. and Falkow,S. (1984) *J. Bacteriol.*, **157**, 330−333.
Caserta,M., Zacharias,W., Nwankwo,D., Wilson,G.G. and Wells,R.D. (1987) *J. Biol. Chem.*, **262**, 4770−4777.
Dente,L., Cesarini,G. and Cortese,R. (1983) *Nucleic Acids Res.*, **11**, 1645−1655.
Gold,L. and Stormo,G. (1987) In Ingraham,J.L. (ed.), *Escherichia coli and Salmonella typhimurium Cellular and Molecular Biology.* American Society for Microbiology, Washington, DC, pp. 1302−1307.
Guerina,N.G., Langermann,S., Clegg,H.W., Kessler,T.W., Goldmann, D.A. and Gilsdorf,J.R. (1982) *J. Infect. Dis.*, **146**, 564.
Guerina,N.G., Langermann,S., Schoolnik,G.K., Kessler,T.W. and Goldmann,D.A. (1985) *J. Exp. Med.*, **161**, 145−159.
Hanson,M.S. and Brinton,C.C., Jr (1988) *Nature*, **332**, 265−268.
Harley,C.B. and Reynolds,R.P. (1987) *Nucleic Acids Res.*, **15**, 2343−2361.
Hohn,B. and Collins,J. (1980) *Gene*, **11**, 291−298.
Klemm,P. (1984) *Eur. J. Biochem.*, **143**, 395−399.
Lindberg,F., Lund,B. and Normark,S. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 1891−1895.
Livey,J., Duggleby,D.J. and Robinson,H. (1987) *Mol. Microbiol.*, **1**, 203−209.
Loeb,M.R., Connor,E.M. and Penney,D. (1988) *Infect. Immun.*, **56**, 484−489.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Pearce,W.A. and Buchanan,T.M. (1980) In Beachey,E.H. (ed.), *Bacterial Adherence.* Chapman and Hall, London, pp. 289−344.
Pearson,W.R. and Lipman,D.J. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 2444−2448.
Pichichero,M.E., Anderson,P., Loeb,M. and Smith,D.H. (1982) *Lancet*, **ii**, 960−962.
Poole,J. and van Alphen,L. (1988) *Transfusion*, **28**, 289.
Reed,K.C. and Mann,D.A. (1985) *Nucleic Acids Res.*, **13**, 7207−7221.
Sable,N.S., Connor,E.M., Hall,C.B. and Loeb,M.R. (1985) *Infect. Immun.*, **48**, 119−123.
Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463−5467.
Slot,J.W. and Geuze,H.J. (1985) *Eur. J. Cell Biol.*, **38**, 87−93.
Turk,D.C. (1985) *J. Med. Microbiol.*, **18**, 1−16.
van Alphen,L., Poole,J. and Overbeeke,M. (1986) *FEMS Microbiol. Lett.*, **37**, 69−71.
van Alphen,L., Poole,J., Geelen,L. and Zanen,H.C. (1987) *Infect. Immun.*, **55**, 2355−2358.
van Alphen,L., van den Berghe,N. and Geelen-van den Broek,L. (1988) *Infect. Immun.*, **56**, 1800−1806.
van Die,I., van Geffen,B., Hoekstra,W. and Bergmans,H. (1984) *Gene*, **34**, 187−196.
van Embden,J.D.A,, van der Donk,H.J., van Eijk,R.V., van der Heide, H.G., de Jonge,J.A., van Olderen,M.F., Osterhaus,A.D. and Schouls, L.M. (1983) *Infect. Immun.*, **42**, 187−196.
von Hippel,P.H., Bear,D.G., Morgan,W.D. and McSwiggen,J.A. (1984) *Annu. Rev. Biochem.*, **53**, 389−446.

Received on July 17, 1989