Genetical and functional investigation of \( fliC \) genes encoding flagellar serotype H4 in wildtype strains of \textit{Escherichia coli} and in a laboratory \textit{E. coli} K-12 strain expressing flagellar antigen type H48

Lothar Beutin*, Eckhard Strauch, Sonja Zimmermann, Stefan Kaulfuss, Christoph Schaudinn, Andrea Männel and Hans R Gelderblom

Abstract

\textbf{Background:} Serotyping of O-(lipopolysaccharide) and H-(flagellar) antigens is a widely used method for identification of pathogenic strains and clones of \textit{Escherichia coli}. At present, 176 O- and 53 H-antigens are described for \textit{E. coli} which occur in different combinations in the strains. The flagellar antigen H4 is widely present in \textit{E. coli} strains of different O-serotypes and pathotypes and we have investigated the genetic relationship between H4 encoding \( fliC \) genes by PCR, nucleotide sequencing and expression studies.

\textbf{Results:} The complete nucleotide sequence of \( fliC \) genes present in \textit{E. coli} reference strains U9-41 (O2:K1:H4) and P12b (O15:H17) was determined and both were found 99.3\% (1043 of 1050 nucleotides) identical in their coding sequence. A PCR/RFLP protocol was developed for typing of \( fliC \)-H4 strains and 88 \textit{E. coli} strains reacting with H4 antiserum were investigated. Nucleotide sequencing of complete \( fliC \) genes of six \textit{E. coli} strains which were selected based on serum agglutination titers, \( fliC \)-PCR genotyping and reference data revealed 96.6 to 100\% identity on the amino acid level. The functional expression of flagellin encoded by \( fliC \)-H4 from strain U9-41 and from our strain P12b which is an H4 expressing variant type was investigated in the \textit{E. coli} K-12 strain JM109 which encodes flagellate type H48. The \( fliC \) recombinant plasmid carrying JM109 strains reacted with both H4 and H48 specific antisera whereas JM109 reacted only with the H48 antiserum. By immunoelectron microscopy, we could show that the flagella made by the \( fliC \)-H4 recombinant plasmid carrying strain are constituted of H48 and H4 flagellins which are co-assembled into functional flagella.

\textbf{Conclusion:} The flagellar serotype H4 is encoded by closely related \( fliC \) genes present in serologically different types of \textit{E. coli} strains which were isolated at different time periods and geographical locations. Our expression studies show for the first time, that flagellins of different molecular weight are functionally expressed and coassembled in the same flagellar filament in \textit{E. coli}. 

Published: 24 January 2005

Received: 09 August 2004

doi:10.1186/1471-2180-5-4

This article is available from: http://www.biomedcentral.com/1471-2180/5/4

© 2005 Beutin et al; licensee BioMed Central Ltd.

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
Background

Bacterial strains belonging to the *Enterobacteriaceae* species *Escherichia coli* are common as commensals in the intestinal flora of humans and warm-blooded animals [1]. Typing systems for identification of related *E. coli* strains were developed in the early 1940ies when it became evident that certain *E. coli* strains were agents of infantile gastroenteritis [2]. In 1944, Kauffmann established the method of serological typing for *E. coli* O- (lipopolysaccharide) and H-(flagellar) antigens which allowed the grouping of *E. coli* strains according to their O:H-types (serotypes) [3]. Serotyping proved to be widely useful for identification of enteropathogenic *E. coli* (EPEC) strains from stools of diarrhoeic infants [4,5] and is successfully employed for characterization of pathogenic *E. coli* strains from both humans and animals [2,3].

The genetic analysis of *E. coli* populations by multilocus enzyme electrophoresis (MLEE) and multilocus sequence typing (MLST) allowed the detection of clonal types of strains which carry specific virulence markers and are associated with disease in humans [6,7]. It was shown that the O:H1 serotype is a good marker for identification of strains belonging to clonal types of pathogenic *E. coli* [6,7]. At present, 176 O- and 53 H-antigens are described for *E. coli* which can occur in different combinations in wildtype isolates of strains [2,3,5,8]. However, the large number of O- and H-antísera which are needed for *E. coli* serotyping and the laborious typing procedure restricts its usage to a few reference laboratories. Therefore, alternative typing methods were developed including molecular characterization of genes coding for the O- and H-antigens in *E. coli* [9-12]. Typing of fliC genes by PCR was successfully employed for characterization of human pathogenic O157:H7 and O26:H11 strains [13,14]. Analysis of the nucleotide sequence of *fliC* genes coding for flagellar antigens H7 and H6 revealed large sequence similarities between strains sharing the same H-type but different O-types [15,16]. Moreover, molecular typing of the *fliC* gene allows H-typing of non-flagellated (non-motile) *E. coli* isolates which cannot be analyzed for their flagella with H-specific antisera [14,15,17].

The flagellar type H4 is frequently occurring in *E. coli* belonging to many different O-groups including strains of shiga toxin-producing *E. coli* (STEC) and extraintestinal pathogenic serotypes [18-20], (K.A. Bettelheim, The VTEC table, May 2003, [http://www.microbionet.com.au/vitectable.htm](http://www.microbionet.com.au/vitectable.htm)). Moreover, a cryptic *fliC*-H4 gene was described to be present and to be spontaneously expressed in *E. coli* strain P12b (O15:H17) which carries another type of flagella called H17 which is not encoded by the *fliC* gene [21,22]. Therefore, we became interested in the genetic variability of flagellar H4 genes in *E. coli* strains belonging to different O-serogroups and pathotypes. We have used the published nucleotide sequence of the *fliC* gene present in the *E. coli* H4 reference strain U9-41 (accession AB028472) to develop a PCR which allows discrimination between the *fliC*-H4 gene variants. Nucleotide sequence analysis of the *fliC* gene was performed on other *E. coli* H4 strains that either showed deviations in the PCR analysis or were reported to harbour allelic types of the H4-*fliC* gene [9] or revealed differences in the agglutination reaction compared to the reference strains. To study the expression of different flagellar H4 types we have cloned their corresponding *fliC* genes and have introduced them into the laboratory *E. coli* K-12 strain JM109 [23]. Expression of recombinant flagella was demonstrated by serotyping and by immuno electron microscopy.

Results

Serological detection of the flagellar H4 antigen in different *E. coli* wildtype host strains

*E. coli* reference strains U9-41 (O2:K1:H4) and P12b (O15:H17) [3] and the *E. coli* K-12 strain JM109 (Orough:H48) (this study), which was used for expression studies were investigated for inhibition of motility in swarm-agar containing 1:600 dilutions of either H4, or H48-antiserum (see Methods). U9-41 and P12b were fully inhibited for their motility in the presence of H4 antiserum derived from strains U9-41 or from P12b but not in the presence of H48 specific antiserum. The *E. coli* K-12 strain JM109 was not inhibited for motility by H4 antiserum but by H48 antiserum. These findings indicate that H4 antiserum specifically inhibited the motility of *E. coli* H4 strains and that the antigenically different flagellar type H17 was not expressed or lost in our P12b isolate, similar as previously described with spontaneously arising variants of P12b [24]. We became interested if other *E. coli* H17 strains would also carry the genes for expression of H4 type flagella as it was described for P12b [21,22,24]. For this, we have investigated five additional *E. coli* H17 strains (872-69, 107-74, 305-78, 870-69 and 327-01) for their serological reaction with different H4 specific antisera and all strains showed specific positive reactions (Table 1). H-serotyping performed with strains from the collection of the Robert Koch-Institute revealed 88 *E. coli* strains which agglutinated with H4 antisera and the results obtained with 10 representative strains are shown in Table 1. All strains agglutinated with both, H4<sub>U9-41</sub> and H4<sub>P12b</sub> antigen, but were not agglutinating with antiserum made against other H-antigens (data not shown). Differences in agglutinating titers between H4<sub>U9-41</sub> and H4<sub>P12b</sub> antiserum were not more than twofold with either strain indicating that both sera were similar for their specificity (Table 1). The strains P7d, E1541-68, 107-74 and 305-78 showed significantly lower agglutination titers with H4 antiserum than did the reference strains U9-41 and P12b, which had been used for production of H4 typing
sera, respectively (Table 1). These findings prompted us to compare all the H4 strains with all other \textit{E. coli} H-types for polymorphisms in the \textit{fliC} gene by HhaI digestion of \textit{fliC}-PCR products as described in the Method section.

\textbf{HhaI-RFLP typing of \textit{fliC} genes in \textit{E. coli} \textit{PCR} products}

\textit{PCR} products obtained with primers \textit{fliC}-1 and \textit{fliC}-2 were digested with HhaI to obtain H-serotype specific RFLP patterns from reference strains for the 53 different \textit{E. coli} \textit{H}-serotypes [3,9]. HhaI-RFLP typing of \textit{E. coli} \textit{fliC} genes coding for flagellar types H1 to H56 revealed individual patterns corresponding to the different H-serotypes (Fig. 1). Flagellar antigens H3, H17, H35, H36, H44, H47, H53, H54 and H55 were reported to be not encoded by \textit{fliC} but by other genes (\textit{flkA}, \textit{fllA}, \textit{flmA} and others) in the corresponding \textit{E. coli} strains and the \textit{fliC} HhaI patterns obtained from these strains do therefore not correspond to their H-serotypes [21,22].

HhaI-RFLP patterns were found conserved among strains sharing the same H-serotype independent of their O-antigen as previously described [9] (data not shown). An exception was found for H-serotypes 2, 8, 18, 19, 21, 33 and 47 in which single strains possessed different HhaI-RFLP patterns when compared with the corresponding H-type reference strain (data not shown). The HhaI-RFLP patterns of different H-types were distinguishable from each other (Fig. 1). The results from HhaI-RFLP typing corresponded with the earlier reports showing that the \textit{fliC} gene in strain P12b codes for flagella of serotype H4. The relationship between \textit{fliC} genes present in the different \textit{E. coli} strains was further investigated by nucleotide sequencing.

\textbf{Nucleotide sequence analysis of the \textit{fliC} genes present in representative \textit{E. coli} H4 strains}

To obtain the entire coding region of the \textit{fliC} gene in \textit{E. coli} strains oligonucleotide primers \textit{fliC}-5 and \textit{fliC}-6 were deduced from the \textit{fliC}-H4 chromosomal region and applied to amplify the corresponding chromosomal regions from \textit{E. coli} strains U9-41 (O2:K1:H4), P12b (O15:H17), U1-41 (O5:K4:H4), P7d (O68:H4), C107-74 (O15:H17) and E1541-68 (O154:H4) as listed in Table 1.

These strains were taken as flagellar type H4 representatives according to previously published results [3,9] and according to the H-serotyping performed in this study. The analysis of the coding sequences of the \textit{fliC} genes present in these strains revealed in all cases a length of 1050 bp. As a control, the \textit{fliC} gene of the strain U9-41 was sequenced and found to be identical to the previously deposited \textit{fliC} sequence (accession AB028472). The identity of the \textit{fliC}-H4 sequences ranges from 97.6% to 100%. The greatest sequence difference to the \textit{fliC} gene coding sequence of strain U9-41 was found in the \textit{fliC} gene of strain P7d (exchange of 20 nucleotides), whereas the \textit{fliC} sequences of strains U1-41 and U9-41 were identical to each other. All deduced flagellins have a length of 349 amino acids. The greatest deviation in the primary structure to the FliC protein of the reference strain U9-41 was again observed for the FliC protein of strain P7d (exchange of 9 aa in a stretch of 349 aa).

The alignment of the deduced FliC proteins of all investigated strains. The FliC protein of strain U1-41 is 100% identical to that of strain U9-41 and therefore not shown. Similarities of the deduced amino acid sequences of the FliC proteins (flagellins) are summarized in Table 2.
HhaI digested fliC PCR products obtained with primers fliC-1 and fliC-2 from E. coli reference strains for 53 different expressed H-types as indicated at the right side. Similarity of restriction fragment patterns was calculated with BioNumerics software and is indicated by the dendrogram on the left side. The flagellar antigens of strains encoding H-types H3, H17, H35, H36, H44, H47, H53, H54 and H55 are not encoded by fliC but by other genes (flkA, flIA, flmA and others) in the corresponding E. coli strains and the fliC HhaI patterns obtained from these strains do therefore not correspond to their H-serotypes [21, 25, 26, 27].

**Figure 1**

HhaI digested fliC PCR products obtained with primers fliC-1 and fliC-2 from E. coli reference strains for 53 different expressed H-types as indicated at the right side. Similarity of restriction fragment patterns was calculated with BioNumerics software and is indicated by the dendrogram on the left side. The flagellar antigens of strains encoding H-types H3, H17, H35, H36, H44, H47, H53, H54 and H55 are not encoded by fliC but by other genes (flkA, flIA, flmA and others) in the corresponding E. coli strains and the fliC HhaI patterns obtained from these strains do therefore not correspond to their H-serotypes [21, 25, 26, 27].
PCR based detection of fliC-H4 specific DNA sequences

Amplification of the fliC genes present in E. coli strains U9-41 and P12b with primers fliC-1 and fliC-2 resulted in the generation of a 953 bp internal PCR product with both strains (Fig. 3, lanes 4+5). The nucleotide sequences of this stretch of DNA of strains U9-41 and P12b were compared for restriction enzymes which cut at different sites in the fliC-H4U9-41 and fliC-H4P12b sequence. HhaI was found to cut at identical sites confirming the results from HhaI RFLP typing (Table 3). In contrast, enzymes HpaII and MboI were found to generate each different restriction fragments from PCR products of the fliC-H4U9-41 and fliC-H4P12b gene, respectively. Both enzymes were taken for RFLP typing of amplified fliC genes (primers fliC-1 and fliC-2) from 88 E. coli strains which showed agglutination reactions with H4 antisera (Table 3). Eighty-six of the 88 strains showed HpaII and MboI restriction profiles which corresponded to the patterns obtained with

Alignment of the deduced FliC (flagellin) sequences from E. coli strains representing the flagellar antigen H4 and its genetic variants: U9-41 (accession BAA85081); C107-54 (accession CAE53943), E1541-68 (accession CAD60547); P12b (accession CAD56695); and P7d (accession CAE53942).

Table 2: Amino acid identity/divergence between the deduced FliC proteins of the six investigated E. coli strains (aligned length 349 aa, no gaps).

|            | U9-41 | U1-41 | C107-74 | E1541-68 | P12b | P 7d |
|------------|-------|-------|---------|----------|------|------|
| Percent divergence |
| U9-41      | 100.0 |       | 99.7    | 99.4     | 98.6 | 97.4 |
| U1-41      | 0.0   | 100.0 | 99.7    | 99.4     | 98.6 | 97.4 |
| C107-74    | 0.3   | 0.3   | 100.0   | 99.1     | 98.3 | 97.1 |
| E1541-68   | 0.6   | 0.6   | 0.9     | 100.0    | 98.0 | 98.0 |
| P12b       | 1.4   | 1.4   | 1.7     | 2.0      | 3.5  |      |
| P 7d       | 2.6   | 2.6   | 2.9     | 2.0      | 3.5  | 1.2  |

From HhaI RFLP typing (Table 3). In contrast, enzymes HpaII and MboI were found to generate each different restriction fragments from PCR products of the fliC-H4U9-41 and fliC-H4P12b gene, respectively. Both enzymes were taken for RFLP typing of amplified fliC genes (primers fliC-1 and fliC-2) from 88 E. coli strains which showed agglutination reactions with H4 antisera (Table 3). Eighty-six of the 88 strains showed HpaII and MboI restriction profiles which corresponded to the patterns obtained with...
Exceptions were made by strains P7d (O68:H4) and P12b (O15:H17) which showed individual restriction patterns which differed from all other H4 strains investigated in this study. (Table 3).

Cloning and expression of fliC-H4 genes in the E. coli K-12 strain JM109

In order to study the functional expression of the fliC-H4 genes in a different genetic background we cloned the corresponding PCR products of strains U9-41 and P12b into the vector pLITMUS38 as described in the Methods section. The fliC coding regions were inserted downstream of the lacZ promoter of pLITMUS38 and the fliC recombinant plasmids were transformed into the laboratory E. coli K-12 strain JM109 [23]. JM109 was serotyped as O-rough:H48, and it showed the same HhaI-RFLP fliC-pattern as the E. coli reference strain P4 (O16:H48) [3] (Fig. 1). The functional expression of the cloned fliC-H4 genes in the JM109 derivative strains TPE1976 (fliC-H4U9-41 clone) and TPE1978 (fliC-H4P12b Clone) was analyzed by tube agglutination with H4 and H48 antisera, respectively. The strains TPE1976 and TPE1978 showed agglutination with H48 and H4 sera whereas the parental JM109 strain reacted only with H48 serum (Table 4). To find out if the flagellins of the fliC recombinant plasmid carrying JM109 strains were co-assembled in all flagella or assembled separately we studied the parental strain JM109 and its fliC-H4 derivative TPE1978 by IEM (Fig. 4B–G). Both strains were found to express 2-4 flagella per cell which appeared morphologically typical as long helical filaments (diameter 18 nm, length up to 20 μm) (Fig. 4A), [29]. The reaction of H48 antiserum with bacteria followed by detection of adsorbed antibodies with anti-

---

### Table 3: PCR/RFLP typing of fliC genes in E. coli H4 strains with restriction enzymes HhaI, HpaII and MboI

| fliC-genotype (prototype strain) | HhaI    | HpaII   | MboI   |
|---------------------------------|---------|---------|--------|
| fliC-H4 (U9-41)*               | 362, 304, 104, 66, 50, 50a, 164d, 16a2d | 296, 240, 225, 159, 33d | 429, 313, 182, 29d |
| fliC-H4 (P7d)b                 | 362, 304, 104, 66, 50, 50a, 164d, 16a2d | 296, 273, 225, 159 | 429, 313, 182, 29d |
| fliC-H4 (P12b)c                | 362, 304, 104, 66, 50, 50a, 164d, 16a2d | 296, 273, 225, 159 | 495, 458 |

a) PCR/RFLP patterns found in strains U9-41, U1-41, E1541-68, C107-74 and in 82 E. coli strains which reacted with H4 antisera. The serotypes of all 86 strains are: O2:K1 (4 strains), O5 (2), O7 (1), O8 (1), O13 (1), O15 (2), O20 (2), O50 (1), O60 (1), O77 (1), O78 (1), O99 (3), O111 (9), O113 (2), O114 (5), O119 (1), O141 (3), O154 (1), O176 (1), O181 (1), O-untypable (9), O-rough (3). Forty-two of the strains belonging to O-groups O60, O113, O114, O141 as well as O-rough and O-untypable strains produced Shiga-toxins.
b) RFLP patterns found with strain P7d (O68:H4)
c) RFLP-patterns found with strain P12b (O15:H17)
d) fragments smaller than 50 bp are not detectable on 2% agarose gels (Fig. 3). Exact fragment sizes were calculated on the basis of nucleotide sequence analysis for each of the restriction enzymes used.

---

### Figure 3

Electrophoretic separation of restriction enzyme digested fliC PCR products (primers fliC-1 and fliC-2) of E. coli strains U9-41 and P12b on 2% agarose: Lanes: 1+8= molecular weight standard; 2 = U9-41 (HpaII); 3 = P12b (HpaII); 4 = U9-41 (undigested); 5 = P12b (undigested); 6 = U9-41 (MboI); 7 = P12b (MboI). Sizes of restriction fragments are listed in Table 2.
rabit-IgG coupled with 10 nm immuno-gold particles showed a specific and homogeneous labelling of flagella present on the surfaces of JM109 (Fig. 4C) and of TPE1978 (Fig. 4B). When H4 serum was used, only flagella of strain TPE1978 became immuno-labelled (5 nm gold particles) (Fig. 4D) and no labeling was observed with JM109 (Fig. 4E), confirming H-serotyping results. Sequential double labeling experiments with H48 and H4 antibodies resulted in staining of all flagella on the surfaces of JM109 (Fig. 4G) and TPE1978 (Fig. 4F). However, both 5 nm (H4 label) and 10 nm (H48 label) gold particles were only bound to flagella of TPE1978 (Fig. 4F) whereas the flagella of JM109 were exclusively labelled with 10 nm gold particles which indicates the H48 antigen (Fig. 4G). These results demonstrate that both H48 and H4 flagellins are co-assembled in the flagella made by the fliC-H48/H4 genes in strain TPE1978.

Discussion
The fliC genes of representative E. coli strains for the 53 different H-types were recently investigated and compared for their nucleotide sequences [21]. Among the H-type reference strains which were not analyzed for their complete fliC gene sequences, were the reference strains for the E. coli H4 (U9-41) and H17 (P12b) flagellar antigen [21,22]. In this work, we performed a complete characterization of the fliC-H4 genes from different E. coli strains with primers which were generated on the basis of the previously published fliC sequence (accession AB028472) of the E. coli H4 reference strain U9-41. The comparison of the fliC gene sequence encoding H4 flagella in strain P12b with the fliC sequences of five other E. coli H4 or H17 strains revealed a high similarity on the DNA and on the amino acid level (Fig. 2 and Table 2). We established a PCR/RFLP typing assay for genotypic investigation of clinical E. coli isolates which reacted with H4 antisera. Genotyping of 88 E. coli strains comprising 20 different O-serogroups (Table 3) revealed that 86 of the strains gave RFLP patterns with HhaI, MboI and HpalI which were indistinguishable from the prototype fliC-H4 gene and only two strains showed alternative patterns. The detection of some genetic variants in the fliC-H4 gene of E. coli strains studied here points to a sequence diversity similar as described previously for the fliC genes of E. coli H6 and H7 strains [15,16]. These results correspond to previous findings indicating that the H4 antigens in different E. coli typing strains are not fully identical [20,24,28].

It was suggested earlier that the strain P12b encodes two flagellins, H4 and H17, which are subject to phase variation for their expression [22] and mutants of P12b could be isolated which expressed only the H4 antigen [24]. Recent studies have demonstrated that the fliC gene of strain P12b encodes flagellar type H4 and it was suggested that the gene for the H17 flagellin is encoded by a locus outside fliC, however the gene responsible for the flagellar type H17 was not identified in the study [21]. In our study, cultures of strain P12b were fully inhibited for motility and swarming in the presence of H4 antiserum but not in the presence of H48 antiserum which was used a non-specific control. This result indicates that H17 type flagella were not expressed or lost from our P12b isolate, similar as previously described with mutant strains of P12b [24].

The functional expression of the cloned fliC-H4(U9-41) and fliC-H4(P12b) genes in the genetic background of E. coli K-12 strain JM109 which shows flagellar serotype H48 confirmed their coding capacity. Since we found coexpression of cloned flagellins with the parental H48 flagellin, we became interested in the composition of flagella made by the fliC recombinant plasmid carrying JM109 strains. Electron microscopy of flagella from JM109 and from the fliC-H4(P12b) recombinant plasmid carrying strain revealed

---

Table 4: H-Agglutination reaction of fliC-H4 recombinant plasmid carrying E. coli K-12 strains

| Strain     | serotype or fliC recombinant JM109 derivative | agglutination with H-specific antisera<sup>a</sup> |
|------------|----------------------------------------------|-----------------------------------------------|
|            |                                              | H4                                      |
|            |                                              | H4<sub>K-12</sub> |
|            |                                              | H4<sub>P12b</sub> |
| U9-41      | O2:K1:H4                                     | <200<sup>b</sup>                      | 12800                          | 12800                          |
| P12b       | O15:H17                                      | <200<sup>b</sup>                      | 6400                           | 12800                          |
| JM109<sup>c</sup> | O-rough:H4                                   | 12800                          | <200<sup>b</sup>                      | <200<sup>b</sup>                      |
| TPE1976    | JM109<sup>d</sup> (pLIITMUS38-fliC-H4<sub>U9-41</sub>)<sup>e</sup> | 12800                          | 12800                          | 12800                          |
| TPE1978    | JM109<sup>d</sup> (pLIITMUS38-fliC-H4<sub>P12b</sub>)<sup>e</sup> | 12800                          | 6400                           | 12800                          |

a) reciprocal value agglutination titers with antisera (the same results were obtained in two separate experiments). H4<sub>K-12</sub> and H4<sub>P12b</sub> indicates the H4 antisera produced with strains U9-41 and P12b, respectively.

b) no agglutination with start serum dilution 1:200

c) E. coli K-12 [23]. d) fliC-H4 gene cloned from strain U9-41
e) fliC-H4 gene cloned from strain P12b
Figure 4

(A) Low power micrograph of *E. coli* strain TPE1978 cells showing the density of the bacterial samples used for indirect IEM and the presentation of flagella (bar length = 1 µm) (B) IEM of strain TPE1978 flagella after incubation with rabbit flagellar H48 antiserum (1:1000) and detection of bound antibody by anti-rabbit-IgG-10 nm gold (1:20), bar length = 100 nm. (C) Strain JM109 flagella after incubation with rabbit flagellar H48 antiserum and detection of bound antibody by anti-rabbit-IgG-10 nm gold. (D) Strain TPE1978 flagella after incubation with rabbit flagellar H4 antiserum (1:1000) and detection of bound antibody by anti-rabbit-IgG-5 nm gold. (E) Strain JM109 flagella after incubation with rabbit flagellar H4 antiserum and detection of bound antibody by anti-rabbit-IgG-5 nm gold. (F) Double-labeling IEM of strain TPE1978 after sequential incubations with rabbit flagellar H4 antiserum and anti-rabbit-IgG 5 nm gold, followed by rabbit flagellar H48 antiserum detected by anti-rabbit-IgG-10 nm gold. Both, 5 nm and 10 nm gold markers are bound at comparable amounts over all flagella present on the bacteria. (G) Double-labeling IEM of strain JM109 after sequential incubations with rabbit flagellar H4 antiserum and anti-rabbit-IgG-5 nm gold followed by rabbit flagellar H48 antiserum and anti-rabbit-IgG-10 nm gold. Only H48 specific (10 nm) gold particles are bound to the flagella of JM109.
no differences between JM109 and TPE1978 showing both a typical helical organization of normal sized flagella on their surface (Fig. 4). Immuno-gold staining of bacteria which were prior incubated with H48 and H4 antiserum revealed high specificity of the rabbit antisera for the flagellar structure (Fig. 4B−G). Single (Fig. 4 B+D) and double labelling (Fig. 4 F) experiments with different sized gold markers demonstrated that all flagella present on the surface of TPE1978 were labelled after incubation with H48 and H4 antiserum. Our results indicate that both H48 and H4 flagellins are coassembled in the flagella made by the fliC-H4p12b recombinant plasmid carrying strain. This finding is surprising in view of the molecular weight size differences found between H48 and H4 flagellin. To our knowledge, the assembling of two different flagellins in the same filament has not yet been demonstrated before. The H48 flagellin of E. coli K-12 (accession AE000285) is a 51.3 kDa protein consisting of 498 amino acids and thus much larger than the 36.3 kDa H4 flagellin which is composed of 349 amino acids. However, both flagellins share conserved N- and C-terminal sequences which are known to be involved in the structural assembly of flagella [29]. H48 and H4 flagellins differ largely for their central regions which are not involved in flagellar assembly and function but which contain flagellar antigenic epitopes [29].

We were able to show that the introduction of an isolated fliC gene in E. coli can change the antigenic properties of the flagella made by this strain. Horizontal transfer of fliC genes may contribute to the diversity of flagellar serotypes by recombination within E. coli recipient strains. The mammalian host immune system is the driving force for continuous selection of new flagellar antigens in E. coli. Published data indicate that both mutation and recombination events in the fliC gene have taken place in the evolution of E. coli flagellar antigens [15,16].

Conclusions
Our fliC sequence data have shown that the flagellar type H4 which is present in E. coli strains of clinical importance covers several genetic variants which are closely related to each other. We have shown for the first time that flagellins of different molecular size are expressed and coassembled into functional flagella in a laboratory E. coli K-12 strain.

Methods
Bacterial strains
The reference strains used for production of antisera for O- and H-typing of E. coli were obtained from the Internationl Escherichia and Klebsiella Centre, Statens Seruminstitut, Copenhagen, Denmark and are described elsewhere [3]. Origin and serotype data on five additional strains with flagellar type H17 are listed in Table 1. A laboratory collection of 88 E. coli isolates originating from humans and animals was investigated by PCR/RFLP typing for fliC-H4 genes. These strains were previously investigated for the O-types and for production of Shiga-toxins (Stx) and were isolated in different countries between 1941 to 2002 [3,19,30] (Table 3). The E. coli K-12 strain JM109 is described elsewhere [23].

Production of E. coli O and H-specific antisera
Rabbit antisera against the different O- and H-antigens of E. coli were prepared according to Ørskov and Ørskov [3]. Antisera for typing of flagellar antigens H4 were produced with reference strains U9-41 (O2:K1:H4) and P12b (O15:H17) [3]. Our strain P12b was found to express its fliC encoded H4 antigen and produced flagellar type H4 (this work).

Motility inhibition test
Expression of flagella and swarming of E. coli strains was tested by inoculating bacteria in tubes containing 10 ml portions of swarm-agar (L-broth + 0.3% agar) as described [3]. Inhibition of motility of E. coli strains in the presence of flagellar-specific antiserum (H4 and H48) was tested in swarm-agar containing a 1:600 dilution of the respective antiserum. Cultures which were inhibited for motility were observed over two weeks for possible switch to motility by phase variation.

Serological typing of H-antigens of E. coli
H-serotyping was performed as described [3]. In brief, bacteria were grown in tubes containing 10 ml 0.3% semi-solid LB-agar [23] for two to three passages. Highly motile bacteria were transferred in LB-medium, incubated 6h at 37°C and inactivated by addition of 0.5% formaldehyde in solution. Agglutination reactions were performed in two fold dilutions of 0.5 ml portions of serum in phosphate-buffered saline pH 7.4 (PBS) [22] with 0.5 ml formalized bacteria in glass tubes which were incubated for 2h at 50°C. Agglutination tests were read by eye immediately after incubation as described [3].

PCR-typing of fliC genes
The oligonucleotide primers fliC-1 (5’ CAA GTC ATT AAT C 3’) and fliC-2 (5’ GAC AT(A/G) TT(A/G) GA(G/A/C) ACT TC(G/C) GT 3’) were used for amplification of internal parts of fliC genes present in the E. coli reference strains as described [9]. The PCR was performed for 25 cycles at 94°C for 60 sec, 55°C for 60 sec and 72°C for 120 sec [9]. PCR products of sizes varying between 950 to 2500 bp were obtained with E. coli reference strains for 53 different H-types [3] (Figure 1). Amplified DNA was digested with HpaI and the resulting restriction fragments were compared on 2% agarose gels. Restriction enzymes HpaI and MboI were used for characterization of fliC-H4 specific PCR products. Gel images were stored digitally and analyzed with BioNumerics soft-
ware, version 2.5 (Applied Maths, Kortrijk, Belgium) for similarity (Dice, complete linkage) (Fig. 1).

**Nucleotide sequence analysis of fliC genes**
Two primers deduced from the published *fliC*-H4 sequence (AB028472) were used for the amplification of the entire *fliC*-H4 coding regions. The PCR was performed for 30 cycles: 30 sec at 94°C, 60 sec at 58.1°C and 90 sec at 72°C with primers fliC-5 (5'-TGA GTG ACC AGA CGA TAA CAG GG-3') and fliC-6 (5'-GGA CGA TTA GTG GGT GAA ATG AGG-3') and yielded a 1243 bp product. PCR products were purified with the QIAquick™ PCR Purification Kit (Qiagen, Hilden, Germany) and separated on an automated DNA sequencer (ABI PRISM® 3100 Genetic Analyzer). The sequences were analysed using the Lasergene software (DNASTAR, Madison, WI, USA) and the Mac Vector software (Oxford Molecular Group, Campell, CA, USA) to assemblings and alignings.

**Nucleotide sequence accession numbers**
The nucleotide sequence of the genomic region of *E. coli* strain P12b (O15:H17) with a size of 1234 bp containing the *fliC* gene for flagellin has been submitted to EMBL database under accession number AJ515904. The coding sequences of the different *fliC* genes from the following strains have been assigned the following accession numbers: AJ605764 for strain U1-41 (O5:H4), AJ605765 for strain P7d (O68:H4), AJ605766 for strain C107-74 (O15:H17) and AJ536600 for strain E1541-68 (O154:H4). The origin of the strains is listed in Table 1.

**Molecular cloning of fliC gene PCR products**
PCR products encompassing the complete coding sequences of the *fliC*-H4 genes were obtained from genomic DNA prepared as described [9] of *E. coli* strains U9-41 and P12b using primers fliC-5 and fliC-6. The amplification products were inserted into the vector pLITMUS38 (New England Biolabs, Beverly, MA, USA) digested with EcoRV. The orientation of the the insert PCR products was determined by using commercially available sequencing primers LITMUS forward 28/38 and LITMUS Reverse 28/38 (New England Biolabs).

**Immuino electron microscopy (IEM) of *E. coli* flagellar antigens**
Motile cultures of *E. coli* strains were produced by repeated passage on semi-solid agar followed by growth in L-Broth as described above. Cultures carrying recombinant pLITMUS38 plasmids were grown in the presence of 100 µg/ml ampicillin. IEM was performed using fresh, non-formalized cultures of motile bacteria. For IEM, aliquots of respective bacterial cultures were diluted 1:2 in PBS pH 7.2 and adsorbed onto glow-discharge treated 400 mesh grids coated with Pioloform and carbon (Wacker Chemie, Munich, Germany) [31]. Grids with adsorbed bacteria were preincubated for 30 min at room-temperature with blocking buffer (0.1 % bovine serum albumine (Sigma, Deisenhofen, Germany) in PBS). Rabbit anti-H48- and anti-H4 hyperimmune sera were diluted 1:1000 in blocking buffer. After conditioning, specimens were incubated for 30 min at room temperature on droplets of the specific, unlabelled antibodies. Non-bound antibody was removed by washing the grids twice for 10 min on blocking buffer. Immuno-specifically bound primary antibodies were detected using anti-rabbit-IgG-gold 5 or -gold 10 nm conjugates (British Bio Cell International Ltd, Cardiff, UK). The conjugates were diluted 1:20 in blocking buffer and reacted for 30 min at room temperature. Unbound conjugate was removed by a sequence of washing steps (two times with blocking buffer for 5 min each; once with PBS for 3 min and a final wash with double destilled water for 3 min) at room temperature. Before negative staining with 1 % uranyl acetate (pH 4.0–4.5), the grids were washed rapidly on 4 droplets of double destilled water. The preparations were analyzed with an EM 10 electron microscope (Zeiss-LEO, Oberkochen, Germany) at an accelerating voltage of 80 kV. To look for different antigenic determinants expressed on the flagella of strains JM109 or TPE1978, double immuno-labelling was performed using H48 and H4 antisera sequentially and two anti-rabbit-IgG-gold conjugates with different sized markers for the detection of the bound primary unlabelled rabbit antibody. Both *E. coli* strains were incubated with rabbit anti-H4 (1:1000) followed by anti-rabbit-IgG-5 nm gold and two washing steps on droplets of blocking buffer for 10 min. The samples were subsequently incubated with anti H48 antibody (1:1000) followed by incubation with anti-rabbit-IgG- 10 nm gold. Removal of surplus conjugate and negative staining were performed as detailed above.

**Author’s contribution**
LB conceived of the study and carried out PCR genotyping and coexpression studies. ES carried out sequence determination and alignments and construction of recombinant plasmids. SZ and SK performed serological assays and PCR genotyping. CS performed analysis of *fliC* recombinant plasmid carrying strains. AM and HG developed the IEM methodology and HG contributed to the data analysis. All authors participated in review and preparation of the final manuscript.

**Acknowledgements**
We are grateful to Ida and Frits Ørskov and to Flemming Scheutz (International Escherichia and Klebsiella Centre, Statens Seruminstitut, Copenhagen, Denmark) for donating *E. coli* serotype reference strains. We thank Bärbel Jungnickel for excellent processing of the electron micrographs.
References

1. Drasar BS, Barrow PA: Intestinal Microbiology. In Aspects of Microbiology Volume 10. Edited by: Schlessinger D, Washington D.C. American Society for Microbiology; 1985.

2. Lior H: Classification of Escherichia coli. In Escherichia coli in domestic animals and humans Edited by: Gyles CL. Wallingford: CAB International; 1994:31-72.

3. Ørskov F, Ørskov I: Serotyping of Escherichia coli. In Methods in Microbiology Volume 14. London: Academic Press; 1984:43-112.

4. Ørskov I, Ørskov F, Jann B, Jann K: Serology, chemistry, and genetics of O and K antigens of Escherichia coli. Bacteriol Rev 1977, 41:667-710.

5. Ewing WH: The Genus Escherichia. In Edwards and Ewing's Identification of Enterobacteriacea Edited by: Ewing WH. New York: Elsevier; 1982:133-148.

6. Caught DA, Levin BR, Ørskov I, Ørskov F, Svanborg EC, Selander RK: Genetic diversity in relation to serotype in Escherichia coli. Infect Immun 1985, 49:407-413.

7. Donnenberg MS, Whitman TS: Pathogenesis and evolution of virulence among pathogenic and enteropathogenic Escherichia coli. J Clin Invest 2001, 107:539-548.

8. Ekland M, Leino K, Sitonen A: Clinical Escherichia coli strains carrying stx genes: stx variants and stx-positive virulence factors. J Clin Microbiol 2002, 40:4585-4593.

9. Machado J, Grimont F, Grimont PA: Identification of Escherichia coli flagellar types by restriction of amplified flic gene. Res Microbiol 2000, 151:535-546.

10. Bottello BA, Bando SY, Trabulsi LR, Moreira-Filho CA: Identification of differences in flagellar antigens in the EPEC O serogroups by PCR-RFLP analysis of the flic gene. J Microbiol Methods 2003, 54:87-93.

11. Prager R, Strutz U, Frucht A, Tschape H: Subtyping of pathogenic Escherichia coli strains using flagellar (H)-antigens: serotyping versus flic polymorphisms. Int J Med Microbiol 2003, 292:477-486.

12. Coimbra RS, Grimont F, Lenormand P, Burguiere P, Beutin L, Grimont PA: Identification of Escherichia coli O-serogroups by restriction of the amplified O-antigen gene cluster (rfb-RFLP). Res Microbiol 2000, 151:639-654.

13. Johnson JR, Stell AL: PCR for specific detection of H7 flagellar variant of flic among extraintestinal pathogenic Escherichia coli. J Clin Microbiol 2001, 39:3712-3717.

14. Zhang WL, Bielaszewska M, Bockemuhl J, Schmidt H, Scheutz F, Karisch H: Molecular analysis of H antigens reveals that human diarrheagenic Escherichia coli O26 strains that carry the eae gene belong to the H111 clonal complex. J Clin Microbiol 2000, 38:2989-2993.

15. Reid SD, Selander RK, Whitcomb TS: Sequence diversity of flagellin (flfG) alleles in pathogenic Escherichia coli. J Bacterial 1999, 181:153-160.

16. Wang L, Rothenmund D, Dur P, Reeves PR: Sequence diversity of the Escherichia coli H7 flfG genes: implication for a DNA-based typing scheme for E. coli O157:H7. J Clin Microbiol 2000, 38:1786-1790.

17. Urdahl AM, Beutin L, Skjerve E, Zimmermann S, Wasteson Y: Animal host associated differences in Shiga toxin-producing Escherichia coli isolated from sheep and cattle on the same farm. J Appl Microbiol 2003, 95:92-101.

18. Ørskov F, Ørskov I: Escherichia coli in extra-intestinal infections. J Hyl (Lond) 1985, 95:551-575.

19. Beutin L, Krause G, Zimmermann S, Kaufuss S, Gleier K: Characterization of Shiga Toxin-Producing Strains Isolated from Humans in Germany over a 3-Year Period. J Clin Microbiol 2004, 42:1099-1108.

20. Ratiner YA, Klimova ZV, Ulisko IN: Use of factor H-sera for discriminating between the serological variants of Escherichia coli flagellar antigens. Zh Mikrobiol Epidemiol Immunobiol 1987, 5:16-20.

21. Wang L, Rothenmund D, Dur P, Reeves PR: Species-wide variation in the Escherichia coli flagellin (H-antigen) gene. J Bacterial 2003, 185:2936-2943.

22. Ratiner YA: Two genetic arrangements determining flagellar antigen specificities in two diphasic Escherichia coli strains. FEMS Microbiol Lett 1985, 29:317-323.

23. Sambrook J: Bacterial Media, Antibiotics and Bacterial Strains. In Molecular Cloning A laboratory Manual Edited by: Sambrook J, Fritsch EF, Maniatis T. Cold Spring Harbor, cold Spring Harbor Laboratory Press; 1989.

24. Ratiner YA: Mutations of E. coli by H-antigens. Zh Mikrobiol Epidemiol Immunobiol 1967, 10:23-28.

25. Ratiner YA: New flagellin-specifying genes in some Escherichia coli strains. J Bacterial 1998, 180:979-984.

26. Ratiner YA: Phase variation of the H antigen in Escherichia coli strain B7327-41, the standard strain for Escherichia coli flagellar antigen H3. FEMS Microbial Lett 1982, 15:33-36.

27. Ratiner YA: Presence of two structural genes determining antigenically different phase-specific flagellins in some Escherichia coli strains. FEMS Microbial Lett 1983, 19:37-41.

28. Ratiner YA: Characteristics of “additional” factors in flagellar antigens of certain serological variants of Escherichia. Zh Mikrobiol Epidemiol Immunobiol 1972, 2:43-48.

29. Macnab RN: Flagella and Motility, In Escherichia coli and Salmonella: cellular and molecular biology Edited by: Curtiss III, R, Ingraham JL, Lin ECC, Brooks Low K, Magasanik B, Reznikoff WS, Riley M, Schaechter M, Umbarger HE. Washington, DC, American Society for Microbiology; 1996:123-145.

30. Beutin L, Geier D, Steinruck H, Zimmermann S, Scheutz F: Prevalence and some properties of verotoxin (Shiga-like toxin)-producing Escherichia coli in seven different species of healthy domestic animals. J Clin Microbiol 1993, 31:2483-2488.

31. Gelderbloem H, Beutin L, Hadiyannidis D, Reupeke H: Rapid typing of pili of Pathogenic Escherichia coli by Dispersive Immunoelectro- n Microscopy, In Rapid Methods and Automation in Microbiology and Immunology Edited by: Habermehl KO. Berlin: Springer-Verlag; 1985:390-400.