Serotype-specific Monoclonal Antibodies against the H12 Flagellar Antigen of *Escherichia coli*

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The flagellar filaments of morphotype E isolates of *Escherichia coli* characteristically possess an apparent helically arranged sheath structure, surrounding the central core of the filament. Re-examination of the type strains of H-serotypes belonging to morphotype E showed that all but serotype H34 possessed the expected morphology. Heterogeneity was observed in both the diameter of filaments from individual morphotype E strains and in the M_r of individual flagellins. There was no apparent correlation between these two features. Monoclonal antibodies (MAbs) of the IgM class were raised against serotype H12 flagella. In Western immunoblotting and agglutination tests, the MAbs recognized the H12 antigen of six isolates with different O:K antigen combinations. The MAbs were H-serotype-specific, with no significant reaction with the H-antigens of other morphotype E strains. The location of the serotype-specific H12 epitope(s) was studied by immunolabelling with colloidal gold markers. The epitope was surface-exposed and appeared to be helically arranged on the flagellar filament. The pattern of colloidal gold labelling was consistent with the possibility that the H12 serotype-specific epitope resides in the apparent sheath structure.

INTRODUCTION

Flagella are organelles of locomotion of bacterial cells. The complete flagellar structure is comprised of three regions, the basal body, the hook, and the flagellar filament (MacNab, 1987). The filament is assembled from many copies of a single protein subunit, flagellin, arranged in approximately eleven longitudinal rows of subunits. The overall effect is a thread-like structure of uniform thickness (approximately 20 nm) and a length in the range of 5–10 μm (MacNab, 1987).

The flagellar filament possesses exposed antigenic determinants (H-antigens), which allow serological distinction between different H-serotypes. There are 53 recognized H-serotypes of *Escherichia coli* (Ørskov & Ørskov, 1984). Of these, 45 H-serotypes can be grouped into six morphological groups (designated morphotypes A–F), based on the appearance of the flagella under the electron microscope (Lawn *et al.*, 1977). Flagella belonging to morphotypes E and F are perhaps the most complex morphologically, due to the presence of an apparent helical sheath structure surrounding the central core of the filament. Eight H-serotypes have flagella with morphotype E filaments and a further 15 H-serotypes exhibit morphotype F (Lawn *et al.*, 1977).

Abbreviation: MAb, monoclonal antibody.

No separate sheath protein has been identified and in morphotype E and F filaments available evidence suggests that the central core and the apparent sheath are both formed from the same flagellin subunits (Lawn, 1977). The M_r values of the flagellin subunits in *E. coli* vary from 37000–69000 and correlate with the diameters of the filaments when compared across the different morphotypes (Lawn, 1977). Serological cross-reactions among H-antigens have been noted and these generally occur between serotypes within the same morphotype (Lawn *et al.*, 1977).

We are interested in the molecular basis for H-antigen diversity in *E. coli*, particularly in strains possessing complex morphotype E and F flagella. The objective of the studies reported here was to investigate the role of the apparent sheath structure in determination of H-antigen specificity in serotype H12 flagellar filaments representative of morphotype E.

METHODS

Bacterial strains and growth media. The bacterial strains used in this study are listed in Table 1. All strains were obtained from the collection of the Collaborative Centre for Reference and Research on *Escherichia coli* (WHO), Copenhagen, Denmark. Cultures were maintained as frozen stocks and grown in Luria broth (LB: Bacto-tryptone, 10 g l⁻¹; Bacto yeast extract, 5 g l⁻¹; NaCl, 5 g l⁻¹) at 37 °C. All strains were passed through semi-solid motility agar (LB containing 0.2% agar) prior to use, to ensure that motile forms were selected.

Isolation of flagella. For electron microscopy and immunogold labelling, flagella with intact basal bodies were isolated using the method described by Johnson *et al.* (1979). Briefly, this procedure involves Triton X-100 lysis of lysozyme and EDTA-treated mid-exponential phase cells. Intact flagella comprising basal body, hook and filament are obtained from DNAase-treated cell lysates, using differential centrifugation.

Flagellin preparations. For analysis of flagellins from a number of bacterial strains, a rapid technique was adopted. Cultures were grown on aluminium trays (40 × 25 cm) containing 500 ml of solid medium. The bacterial growth was scraped off and resuspended in 150 ml 0.1 M-HEPES/NaOH buffer, pH 6.8. All subsequent steps were done at 4 °C. Flagella were removed from the cells by treatment for 1–2 min in a Waring blender. The cells and large membrane fragments were removed from suspension by successive centrifugation steps at 7500 g for 15 min and 9500 g for 15 min, respectively. Flagella and remaining membrane contaminants were harvested from the cell-free supernatant fraction by ultracentrifugation at 200000 g for 1 h. The pellet was resuspended in approximately 0.5 ml 0.1 M-HEPES/NaOH, pH 6.8. Flagellar filaments were converted to flagellin monomers by heating at 90 °C for 10 min. Purified flagellin was then obtained following centrifugation in a Beckman Airfuge at 120 000 g for 20 min, which removed membrane contaminants as a pellet and yielded highly purified flagellin in the supernatant fraction. The degree of purity increased when the supernatant was subjected to a second heating and Airfuge centrifugation cycle. The preparations were essentially free of contaminants as judged by SDS-PAGE and

Table 1. *E. coli* strains used in this study

Strain	Serotype	Reference strain H-serotype	Flagellin M_r *	Filament diameter (nm)†
Su 1242	O2:K2:H1	H1	67000	21
U5-41	O1:K1:H7	H7	67000	19
Bi 316-42	O9:K9:H12	H12	66000	21
K42	O45:K1:H23	H23	67000	19
BP 12665	O86:K?:H34	H34‡	67000	23
4106-54	O52:K?:H45	H45	61000	19
2147-59	O6:K13:H49	H49	60000	19
C218-70	O8:K50:H51	H51	65000	17
E69	O9:K30:H12		66000	21
E47a	O25:K19:H12		66000	21
U12-41	O49:K?:H12		66000	21
P10a	O71:K?:H12		66000	21
H40	O87:K?:H12		66000	21

* Mean of three or more determinations; in each case, the determinations varied ± 1000 .

† Mean of measurements from 10 or more separate filaments; in each case, the determination varied ± 0.1 nm.

‡ Morphology differs from the other strains listed (see text).

NB Lawn (1977) has reported analysis of flagella from strains Su 1242 (diameter 24 nm; M_r 60000), U5-41 (23 nm; 61000) and Bi 316-42 (22 nm; 62000).

Coomassie brilliant blue staining. Protein contents were determined by the Lowry method. The yield of flagellin depended on the strain examined. Routinely 3–8 mg of purified flagellin was obtained from a single tray of culture.

Monoclonal antibody (MAb) production. Two female Balb/c mice (8–10 weeks old) were each immunized subcutaneously on day 1 with 100 µg of purified flagella, suspended in Freund's incomplete adjuvant. Strain E69 was the source of the antigen. On day 10, 50 µg of flagella was administered intravenously. Fusions were done on day 13. Spleen cells from immunized mice were fused with NS-1 murine myeloma cells following the procedure detailed by Lam *et al.* (1987b). Positive hybridomas were identified by enzyme-linked immunosorbent assay (ELISA) with flagella as antigen. Wells were coated using flagella (5 µg ml⁻¹) in carbonate buffer (Voller *et al.*, 1976). The blocking agent was 3% BSA (Sigma) in phosphate-buffered saline (PBS) (Voller *et al.*, 1976). Goat anti-mouse F(ab)₂ conjugated to alkaline phosphatase (Helix Biotech) was used with *p*-nitrophenyl phosphate substrate in the detection system. Positive hybridomas were cloned by limiting dilution. All further experiments were done using hybridoma supernatants. MAb class and subclass were determined using the mouse typer kit from Bio-Rad.

SDS-PAGE. Flagellin preparations were analysed by SDS-PAGE according to the method of Laemmli (1970), using resolving gels containing 10% (w/v) acrylamide and 4% stacking gels. Samples were dissociated in Laemmli sample buffer by heating at 95–100 °C for 10 min immediately before electrophoresis. Protein *M_r* standards were obtained from Bio-Rad. Approximately 1 µg of purified flagellin was loaded per lane.

Western immunoblotting. Flagellin samples were transferred from SDS-PAGE gels to nitrocellulose using modifications (Burnette, 1981) of the method originally described by Towbin *et al.* (1979). Immunoblots were developed using peroxidase-coupled, goat anti-mouse IgG and IgM (Helix Biotech). The colour reagent was 4-chloro-1-naphthol (Sigma).

Slide agglutination reactions. Slide agglutination reactions were performed using overnight cultures. Bacterial growth was scraped from a plate using a sterile toothpick and resuspended in a 0.01 ml drop of saline on a glass microscope slide. Hybridoma supernatant (0.001 ml) was added and mixed with the cell suspension. Clumping of bacterial cells, due to a positive reaction, was evident in less than 20 s.

Preparation of colloidal gold markers. Colloidal gold markers, 12.5 nm in diameter, were coupled to diaminoethane-derivatized dextran using the methods described in detail by Lam *et al.* (1987a). The marker was then coupled to Protein A (Boehringer Mannheim) as described by Hicks & Molday (1986).

Colloidal gold labelling. Copper grids (100 mesh) were coated with carbon and formvar and treated with 0.1% peptone as a wetting agent. Flagella samples contained in a drop of PBS were added to the grid and allowed to dry. The grids were blocked using 3% (w/v) bovine serum albumin in PBS for 15 min and the grid was then washed by inverting on successive drops of PBS. MAb H12-1 (0.05 ml) was added to the grid which was then incubated in a moist chamber at room temperature for 30 min. The grid was then washed exhaustively over drops of PBS containing 0.5% BSA. A second antibody (0.05 ml goat anti-mouse IgM; Cappel Worthington Biochemicals) was then added and allowed to react at room temperature for 30 min. The grid was again washed extensively before addition of the Protein-A-coupled gold marker (0.08 ml). After incubation at room temperature for a further 30 min, the grid was washed and negatively stained. Controls included samples where either the first or second antibody were omitted.

Negative staining and electron microscopy. Samples were negatively stained by inverting the grids over a drop of 0.5% ammonium molybdate, pH 7.0, for 5 s. Use of a negative stain at neutral pH avoided problems with dissociation of flagellar filaments seen with uranyl acetate staining (Lawn *et al.*, 1977). Excess stain was removed using filter paper. The grids were examined in a Philips 300 transmission electron microscope operating at 60 kV.

RESULTS

Production and specificity of anti-H12 MAbs

Hybridomas from fusion experiments were screened initially by ELISA with intact H12 flagella as antigen. Intact flagella were used in an attempt to ensure that only MAbs recognizing surface-exposed epitopes on the flagellar filaments were selected for further study. More than 100 clones were obtained from each of two fusion experiments and three MAbs (designated H12-1, H12-2 and H12-3) were studied further. These three hybridomas were selected since they yielded tissue culture supernatants with high titres of secreted MAb (*A*₄₀₅ greater than 0.1 at a 10⁻⁸ dilution in ELISA); these supernatants were the source of MAb in all subsequent experiments. All the MAbs analysed belonged to class IgM and their reactivities were, on subsequent analysis, indistinguishable. Only the results obtained for MAb H12-1 are reported below.

The serotype specificity of the MAbs was determined by slide agglutination reactions and by Western immunoblotting. All three MAbs gave positive results in slide agglutination tests with the homologous strain (*E. coli* E69). When tested against the H12 type strain (Bi 316-42) and

Fig. 1. SDS-PAGE and Western immunoblotting of flagellins from morphotype E strains of *E. coli*. Flagellins prepared from H12 isolates are shown in (a). Strain numbers are indicated above the appropriate lanes. In (b), an immunoblot from the same gel was probed using MAb H12-1. In (c), the flagellins from each of the morphotype E H-serotype reference strains were analysed by SDS-PAGE. The lanes are labelled according to H-serotype. The immunoblot from the same gel, probed using MAb H12-1, is shown in (d). Flagellin samples were prepared and analysed as described in Methods. Each lane contained approximately 1 μ g protein and only the relevant portion of the gel is shown. For details of strains and flagellin M_r , see Table 1.

against five other H12 serotype strains (E69, E47a, U12-41, P10a and H40), the MABs again gave positive slide agglutination results. None of the MABs agglutinated other H-serotype strains belonging to morphotype E. Western immunoblotting was also used to evaluate the specificity of the MABs, in order to check for weak cross-reactions which might not be detectable by agglutination. MAB H12-1 gave a positive reaction against the flagellins of six different H12-serotype strains (Fig. 1a, b). Only the flagellin band was recognized by this MAB regardless of whether purified flagellin, crude flagellar preparations or membrane fractions were probed (results not shown). When H12-1 MAB was used to probe the flagellins from the eight H-serotype reference strains belonging to morphotype E (Fig. 1c, d), only the H12 antigen was

Fig. 2. Morphology of morphotype E flagellar filaments and immunolabelling of the serotype-H12-specific antigenic epitope. (a, c) Negatively stained serotype H12 filaments, typical of morphotype E. (b) Filaments from serotype H34. (d, e) Immunogold labelling of serotype H12 flagellar filaments. In (e), the edges of successive periodic loops of the apparent sheath, labelled with gold, are indicated by arrows. Bars, 40 nm.

clearly detected. A very slight reaction was consistently seen against the H45 flagellin. This reaction was so weak that even with overloading of antigen, the reaction was not visible in photographs of the resulting immunoblots. An H-serotype strain representing each of the other morphotypes was also tested (results not presented) and no reaction was observed with MAb H12-1 in either agglutination or Western immunoblot tests. The strains tested were *E. coli* U9-41 (serotype H4, morphotype A), U4-41 (serotype H5, morphotype B), P4 (serotype H48, morphotype C), Ulla-44 (serotype H21, morphotype D) and 5306-56 (serotype H46, morphotype F).

Morphology of H12 and H34 flagellar filaments

Although the flagellar filaments of morphotype E have been reported to be morphologically identical (Lawn *et al.*, 1977), analysis of the different flagellins showed some heterogeneity in M_r (Fig. 1c; Table 1). In order to ensure that the filaments analysed were indeed morphotype E, samples were examined by electron microscopy: flagella from seven of the eight morphotype E strains possessed similar morphology. In typical morphotype E strains, exemplified by serotype H12 (Fig. 2a, c), the apparent sheath is readily visible as a structure which appears to completely envelope the central core of the filament. The individual helically arranged loops of the apparent sheath are not readily resolved in typical micrographs. Diagrammatic representations have been presented elsewhere by Lawn *et al.* (1977). One strain, the type strain for H34, produced flagellar filaments with a slightly different morphology (Fig. 2b). In this isolate, the sheath structure comprised larger and more readily visible loops at the edge of the filament and the periodicity of the loops was also different to that of the other morphotype E strains. No filaments devoid of the sheath structure were observed in any of the samples analysed and the morphology for a given isolate was independent of growth conditions.

Electron microscopy also demonstrated that the individual filaments from the different

morphotype E H-serotypes were heterogeneous in terms of their diameters (Table 1). No correlation was found between filament diameter and flagellin M_r .

Antigenic epitopes of H12 flagellar filaments

In an attempt to localize the antigenic epitope on the H12 flagellar filaments, MA b H12-1 was used as a probe coupled with immunogold labelling. As shown in Fig. 2(d), the gold marker specifically labelled the surface of the filaments. It was necessary to reduce the staining of the filament in gold-labelling experiments, in order to make the probe clearly visible. As a result, details of the surface structure of the filament could not be resolved in gold-labelled specimens. Interestingly, the gold marker also labelled the distal tip of the flagellar filament or filament fragments (Fig. 2d). Close examination of the gold-labelled filaments (Fig. 2e) showed many areas in which the gold marker followed a helical path around the filament and the periodicity of the looped structure at the surface of the filament was clearly visible. In Fig. 2(e), successive loops are indicated by arrows.

DISCUSSION

Flagellar filaments from morphotype E strains of *E. coli* are characterized by an apparent helical sheath structure with a 10 nm edge repeat (Lawn *et al.*, 1977). In this report we have examined representative H-serotype strains possessing morphotype E flagella. On re-examination, the filaments from the H34 type strain were found to be slightly different in morphology, when compared to other morphotype E representatives. The H34 filaments resembled more closely the morphology expected for morphotype F (Lawn *et al.*, 1977). The reason for this inconsistency remains unclear as the strain used in this and previous (Lawn *et al.*, 1977) reports is the same. In the original report (Lawn *et al.*, 1977), flagella were fixed in formalin prior to staining in order to minimize the dissociation of acid-labile filaments in acidic uranyl acetate. To avoid these problems, we have used ammonium molybdate at neutral pH for negative staining. Differences in H34 flagellar filament morphology between these studies may therefore result from altered staining procedures and the absence of formalin fixation. These same differences in technique may explain differences in filament diameter in several morphotype E flagella (see Table 1 and footnote) when compared to the values reported by Lawn (1977).

Examination of filament diameters and flagellin M_r values demonstrated heterogeneity among the various representative strains of morphotype E. No correlation was evident between these two measurements, even though it has been shown previously that there is a correlation between filament diameter and flagellin M_r when different morphotypes are considered (Lawn, 1977). The M_r values of the flagellins from different morphotypes range from 37000 in morphotype A to 69000 in the unique morphotype represented by flagella of serotype H36. In comparison, the range of flagellin M_r values within morphotype E is from 60000 to 67000. It is possible, although unlikely, that inaccuracies in measurement of filament diameters, coupled with the limited heterogeneity in flagellin M_r , obscures any relationship within the group. Indeed, Lawn (1977) also examined three representative strains of morphotype E and again within those particular samples, the correlation was not evident. It therefore appears from these results that the diameters of flagellar filaments in individual morphotype E strains do not depend simply on the M_r of the component flagellin subunits. Differences in M_r described in this communication when compared to the previous report may be ascribed to the use of slab gels rather than tube gels and calibration with more markers of known M_r .

Cross-reactions between different H-serotypes have been reported and these often occur between serotypes with morphologically identical flagellar filaments (Lawn *et al.*, 1977). A common cross-reaction is between serotypes H1 and H12 (Ewing, 1986; Ørskov & Ørskov, 1984). The MAbs reported here reacted only with H12 and not with H1 flagellin. Although a slight reaction was observed with H45 flagellin in Western blots, this reaction was weak and insufficient to give a cross-reaction in agglutination experiments. Thus, the MAbs demonstrated precise specificity and may prove to be a useful alternative to absorbed polyclonal sera in H-serotyping.

The epitope recognized by the H12 serotype-specific MAb appears to reside in the apparent sheath structure of the filament, with the gold marker labelling an epitope with the helical and periodic features expected of the sheath. The gold marker was exclusively associated with the filament and no labelling of the basal body was seen in any specimen (results not shown). In some specimens (Fig. 2c, d), free distal tips of filaments were evident. It is not clear whether these tips represent the filament end occurring *in vivo*, or a product of fragmentation during preparation. Interestingly, the gold probe gave extensive labelling of the tips of filaments, suggesting that even at the tips the central filament core may be covered by H12-specific epitope. In terms of antigenic diversity and strategies to escape host immune response, such an arrangement would make sense. It is likely that the central core of the filament contains conserved regions of the flagellin, as this region must fulfil the same function (i.e. assembly into a functional filament) in all H-serotypes. Accordingly, a high degree of homology has been reported between both the amino- and carboxy-termini of flagellins from various bacterial sources (Kuwayama *et al.*, 1986). It would be a selective advantage to the bacterium to keep these conserved regions of the filament in a form which is inaccessible to antibody. In *Salmonella*, the antigenic (and surface exposed) region may reside towards the centre of the flagellin amino acid sequence (MacNab, 1987).

With the isolation of H12-serotype-specific MAbs and the indication that the corresponding epitope resides in the apparent sheath structure, it may now be possible to begin to define the antigenic domains within flagellin subunits and the organization of these epitopes when the subunits are assembled into flagellar filaments. Such studies should lead to a better understanding of the molecular basis for antigenic diversity in the flagellar antigens of *E. coli*.

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