Identification of a common enterobacterial flagellin epitope with a monoclonal antibody

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A monoclonal antibody (mAb), designated 15D8, was produced from BALB/c splenocytes of mice injected with *Escherichia coli* flagella. ELISA of motile cells, non-motile cells and partially purified flagellin proteins showed that the mAb reacted specifically with flagella of *E. coli* and with other members of the family Enterobacteriaceae. Western immunoblot analyses of enterobacterial flagella or cell extracts demonstrated that the antibody reacted with a single protein species in the extracts which was identical in size to purified flagellin. The antigenic determinant for this antibody appears to be surface exposed and linear in configuration, since the antibody reacted with native flagella and flagella which had been denatured. This antibody was also used to demonstrate that although the flagella proteins are heterogeneous in size, at least one epitope is highly conserved.

Introduction

The flagellar filament is a unique structure and is the major component of bacterial flagella (Joys, 1988; Macnab, 1987). Unlike the eukaryotic flagellum, which is relatively large and contains many proteins, the bacterial filament is much smaller in size and composed of several thousand copies of a monomeric protein, flagellin (Iino, 1977; Macnab, 1987). The M_r of flagellin varies greatly amongst bacteria, ranging from 28000 in some *Bacillus* species to over 60000 for some members of the family Enterobacteriaceae (Ibrahim *et al.*, 1985a; Joys, 1988; Kondoh & Hotani, 1974; Kuwajima *et al.*, 1986; Lawn, 1977; McDonough & Smith, 1976). Despite this variation in M_r , nucleic acid and peptide analyses of flagellin from many genera show considerable homology and sequence conservation (Homma *et al.*, 1987; Joys, 1985; Kuwajima, 1988a, b; Szekely & Simon, 1983; Wei & Joys, 1985).

Serological studies on flagella are important clinically for classification and identification of bacteria. For instance, the epitope for the IgA monoclonal antibody (mAb) MOPC467 is highly conserved in *Salmonella* species. This antibody reacts with most of the 1800

serotypes of *Salmonella* (Smith & Potter, 1976), including both phase I and phase II flagellar types (Smith & Jones, 1983). Most other closely related and flagellated bacteria in the family Enterobacteriaceae do not react with the antibody (Robison *et al.*, 1983), so that MOPC467 is useful for the identification of *Salmonella* species.

Both the flagellar filament and the monomeric flagellin protein are potent immunogens. However, not all antigenic epitopes present on native flagella are present on the monomeric proteins (Ibrahim *et al.*, 1985c; Joys, 1988). Serological studies of enterobacterial flagellin have revealed the presence of numerous major and minor antigenic components, including conformational and linear epitopes, which display varying degrees of conservation (Ada *et al.*, 1964; Langman, 1972). Ibrahim *et al.* (1985b) used native *Salmonella* flagella to produce polyclonal antisera which were specific for *Salmonella* serotypes. However, the same antisera were broadly reactive with all *Salmonella* serotypes and other enterobacterial flagella if the flagellar filaments were denatured prior to testing. Unfortunately, it is unclear whether the same antibodies are responsible for both reactivities.

To elucidate whether the structurally conserved flagellar region is also antigenically conserved, we prepared mAbs against *Escherichia coli* flagella. The antibodies were tested for reactivity with different genera from the family Enterobacteriaceae to determine whether the flagella of these organisms are related

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Abbreviation: mAb, monoclonal antibody.

antigenically. We also wanted to determine whether the conserved epitope could be found on intact flagella or whether the flagella require denaturation to expose the primary amino acid sequence.

Methods

Bacterial strains. The organisms used in this study are listed in Table 1. They included 109 *Escherichia coli* strains from clinical and animal sources, and from food and water, six clinical isolates of various *Salmonella* species, and 140 Enterobacteriaceae and 20 Pseudomonaceae that were isolated from water samples collected from the eastern and midwestern sections of the United States. The isolates were identified using the Micro-ID (Organon Teknika) or the API20E

Table 1. Source of bacteria

Bacterium	Strain	Source	Reference*
<i>Citrobacter freundii</i>	33128	Clinical	ATCC
<i>Edwardsiella tarda</i>	15947	Clinical	ATCC
<i>Edwardsiella tarda</i>	CW4	Water	Environmental
<i>Enterobacter aerogenes</i>	13048	Clinical	ATCC
<i>Enterobacter aerogenes</i>	Assorted	Water	Environmental
<i>Enterobacter agglomerans</i>	Assorted	Water	Environmental
<i>Enterobacter cloacae</i>	13047	Clinical	ATCC
<i>Enterobacter cloacae</i>	Assorted	Water	Environmental
<i>Enterobacter gergovia</i>	Assorted	Water	Environmental
<i>Escherichia coli</i>	EC1-72	Food, Water	See O & S
<i>Escherichia coli</i>	1-37		H
<i>Escherichia coli</i>	Assorted	Water	Environmental
<i>Hafnia alvei</i>	Assorted	Water	Environmental
<i>Klebsiella oxytoca</i>	Assorted	Water	Environmental
<i>Klebsiella pneumoniae</i>	23357	—	ATCC
<i>Klebsiella pneumoniae</i>	Assorted	Water	Environmental
<i>Proteus mirabilis</i>	25933	Clinical	ATCC
<i>Proteus mirabilis</i>	Assorted	Water	Environmental
<i>Proteus vulgaris</i>	13315	—	ATCC
<i>Pseudomonas aeruginosa</i>	14207	Clinical	ATCC
<i>Pseudomonas fluorescens</i>	13525	Water	ATCC
<i>Pseudomonas</i> spp.	Assorted	Water	Environmental
<i>Salmonella arizonae</i>	O8	Water	Environmental
<i>Salmonella gallinarum</i>		Clinical	S
<i>Salmonella paratyphi</i> B		Clinical	S
<i>Salmonella tennessee</i>		Clinical	S
<i>Salmonella typhi</i>		Clinical	S
<i>Salmonella typhimurium</i>		Clinical	S
<i>Salmonella worthington</i>		Clinical	S
<i>Serratia liquefaciens</i>	Assorted	Water	Environmental
<i>Serratia marcescens</i>	8100	—	ATCC
<i>Serratia marcescens</i>	Assorted	Water	Environmental
<i>Serratia rubidea</i>	Assorted	Water	Environmental
<i>Shigella sonnei</i>	9290	—	ATCC
<i>Shigella</i> sp.	Assorted	Water	Environmental
<i>Yersinia enterocolitica</i>	MC23	Water	Environmental
<i>Yersinia enterocolitica</i>	O8	—	—

*ATCC, American Type Culture Collection, Rockville, MD, USA; H, P. A. Hartman, Iowa State University, Ames, IA, USA; O & S, H. Ochman & R. K. Selander (1984) (University of Rochester, Rochester, NY, USA); S, B. Swaminathan, Purdue University, W. Lafayette, IN, USA.

system (API Analytab Products). All media used were commercial products and prepared according to the manufacturer's specifications.

Flagellin isolation. Motile isolates of bacteria were obtained by two passages on brain heart infusion (BHI)/0.4% agar plates at 35 °C. Motility was confirmed by microscopic examination of sample wet mounts. Motile cells induced from non-motile isolates were collected after three passages through U-tubes containing BHI medium plus 0.4% agar.

Bacterial flagellin was isolated using a procedure obtained from P. Matsumura, University of Illinois, USA. Briefly, motile cells were grown overnight in nutrient broth containing 0.5% NaCl and then harvested by centrifugation. The cell pellet was resuspended in phosphate-buffered saline (PBS; 10 mM-sodium phosphate, 150 mM-NaCl, pH 7.2), and the heavy cell suspension was mixed with a Waring blender for 1 min to shear the flagellar filaments. After removing the bacterial cells by low-speed centrifugation, the supernatant fluid was centrifuged at 77000 g for 3 h (Beckman SW41-Ti swinging bucket rotor) to pellet the flagellar protein. The pellet of partially purified flagellin protein was resuspended in PBS overnight and then stored at -20 °C.

mAb production and analyses. Anti-flagellin mAbs were produced using standard methods previously described (Sugawara *et al.*, 1983). Briefly, flagellin proteins from *E. coli* strains EC8 and EC51 were injected into 8-week-old BALB/c mice as an emulsion of 10 µg of proteins in Freund's complete adjuvant. The mice were boosted 11 and 40 days later with an additional 10 µg of the same flagellin protein in Freund's incomplete adjuvant; 3 d after the final boost, spleen cells were prepared for fusion with SP2/O myeloma cells.

The mAbs obtained were screened by ELISA using Immulon II Microtiter plates. The plate wells were coated overnight at 4 °C with 50 µl of antigen, which consisted of either flagellin protein at a concentration of 2 µg ml⁻¹ in 50 mM-sodium carbonate buffer, pH 9.5, or a bacterial cell suspension prepared to an OD₅₅₀ of 0.20 in 50 mM-carbonate buffer. After antigen coating, the wells were washed with PBS, and blocked with 200 µl of 3% (w/v) BSA/PBS. The blocking step and all subsequent incubations were for 1 h at 35 °C. Following BSA blocking, 50 µl of hybridoma supernatant fluids or appropriate dilutions of mouse ascites fluids in 1% (w/v) BSA/PBS were added to the wells. The negative control mAb MCA14.2 was prepared against *Neisseria meningitidis* lipopolysaccharide (Sugawara *et al.*, 1983). To detect the antigen-antibody complexes, the wells were treated with 50 µl of peroxidase-conjugated goat anti-mouse IgG used at a 1:1000 dilution in 1% (w/v) BSA/PBS. After each step, unbound reagents in the wells were removed by repeated washing with PBS containing 0.05% Tween 20. The peroxidase enzyme substrate, 2,2'-azino-di(3-ethylbenzylthiazoline sulphonate), was used at a volume of 100 µl per well; after 30 min at room temperature the absorbance was determined using a Titertek Multiskan reader with a 414 nm filter.

Isotypes of the mAbs were determined by ELISA using an antibody isotyping kit (Zymed).

Gel electrophoresis. Electrophoresis of flagellin proteins was done in SDS-polyacrylamide gels in the Tris/glycine discontinuous buffer system (Laemmli, 1970). A 3% (w/v) acrylamide stacking gel was used with a 10% (w/v) acrylamide separating gel. Approximately 5 to 10 µg of each partially purified flagellin preparation in 20 µl of sample buffer (0.12 M-Tris/HCl, pH 6.8; 5%, w/v, SDS; 2%, v/v, 2-mercaptoethanol; 25%, v/v, glycerol; and 0.25% bromphenol blue dye) was boiled for 15 min then loaded into the gel lane. Protein M_r markers (Bio-Rad) were treated similarly. Following electrophoresis, gels were stained for 18 h with 0.025% Coomassie brilliant blue G-250 in 25% (v/v) 2-propanol/10% (v/v) acetic acid, destained for 24 h with 0.0025% Coomassie blue in 10% (v/v) 2-propanol/10% (v/v) acetic acid, and then stored in 10% (v/v) acetic acid solution.

Immunoblotting. Proteins fractionated on SDS-polyacrylamide gels were electrophoretically transferred onto nitrocellulose papers. The transfer was done in Tris/glycine/methanol buffer (25 mM-Tris/HCl, pH 8.3; 192 mM-glycine; 20%, v/v, methanol) at 0.6 A for 2 h with a LKB 2005 Transphor unit. To assay for the presence of specific proteins, the nitrocellulose membranes were first treated overnight with 3% (w/v) BSA/PBS to reduce non-specific binding. Next, hybridoma supernatant or ascites fluids diluted in 1% (w/v) BSA/PBS were incubated with the blots for 2 h at 35 °C to allow antibody to bind to specific proteins. To detect the antigen-antibody complexes, horseradish peroxidase conjugated to goat anti-mouse IgG was diluted 1:1000 in 1% (w/v) BSA/PBS and incubated with the blots for 2 h at 35 °C. Following each step, unbound reagents were removed by four washes, each 15 min, with PBS containing 0.1% Triton X-100. The blots were finally treated for 30 min at room temperature with a precipitable substrate solution consisting of 0.02% 4-chloro-1-naphthol, 10% (v/v) methanol and 0.01% hydrogen peroxide in PBS. The developed blots were rinsed with PBS and dried at room temperature.

Results

Preparation of anti-flagella mAbs

Flagellar protein from two *E. coli* strains was used for the production of hybridomas. Of the 2318 colonies obtained and assayed for antibody by ELISA, 227 produced antibody against *E. coli* EC8 and/or EC51 flagella preparations. Seventy-three hybridomas remained stable producers of antibody after another 10 d, and six were selected for cloning by limiting dilution. The antibodies from the clones showed strong reactivity to the *E. coli* flagellar antigens without cross-reactivity to the non-enteric bacteria (*Pseudomonas* strains), and were produced as ascites fluids.

Antibody specificity

Five of the six antibodies were examined by ELISA for reactivity with natural isolates from food and water, and the clinical and laboratory isolates. Two of the mAbs were highly specific, reacting with only five strains of *E. coli*. Two were intermediate in reactivity, reacting with approximately 40 to 60 *E. coli* isolates and a few *Enterobacter* and *Serratia*. One antibody designated 15D8 (IgG₁, κ) showed cross-reactivity with the majority of genera in the Enterobacteriaceae with the exception of *Klebsiella* and *Shigella* (Table 2).

To determine whether the cross-reactions of mAb 15D8 with the other genera of Enterobacteriaceae were due to binding to flagella or another component of the organism, crude cell lysates and purified flagella were fractionated by SDS-PAGE. Total cellular extracts of *Enterobacter*, *Salmonella*, *Hafnia* and *Serratia* yielded only a single protein which reacted with mAb 15D8 by immunoblotting (Fig. 1). Similarly, *E. coli* lysates yielded only one reactive protein (data not shown). In each case,

Fig. 1. Western immunoblot of flagellin and cell extracts of bacterial isolates using mAb 15D8. The samples were: *Enterobacter cloacae* NJ4 (A); *Salmonella arizonae* O8 (B); *Hafnia alvei* W12 (C); and *Serratia rubidea* O10 (D). For each isolate, flagellin protein was loaded in lane 1, and total crude cell extracts in lane 2. M_r markers (lane S) comprised (from top to bottom): phosphorylase B (M_r 97000); BSA (65000); ovalbumin (45000); carbonic anhydrase (31000); and trypsin inhibitor (20000).

Table 2. *ELISA of mAb 15D8 on environmental, clinical and laboratory isolates*

Fresh cultures of each of the isolates were prepared as a suspension of whole bacteria and used to coat ELISA plates. Isolates showing twofold or greater A_{414} readings with 15D8 as compared to the negative control antibody MCA14.2 were considered positive.

Bacteria	Number tested	Number positive
<i>Citrobacter freundii</i>	1	1
<i>Edwardsiella tarda</i>	2	2
<i>Enterobacter</i> spp.	33	14
<i>Escherichia coli</i>	165	99
<i>Hafnia</i> spp.	6	1
<i>Proteus</i> spp.	8	7
<i>Salmonella</i> spp.	7	7
<i>Serratia</i> spp.	12	9
<i>Yersinia enterocolitica</i>	2	2
<i>Klebsiella</i> spp.	19	0
<i>Pseudomonas</i> spp.	22	0
<i>Shigella</i> spp.	4	0

the antibody-reactive protein species was identical in size to the flagellin protein isolated for each strain. No antibody-reactive protein species were detected in the immunoblot analyses of cell lysates from *Shigella*,

Fig. 2. SDS-PAGE of enterobacterial flagellin proteins. Pre-stained M_r markers (lane F) comprised (from top to bottom): phosphorylase B (M_r 97 000); BSA (65 000); ovalbumin (45 000); and carbonic anhydrase (31 000). The samples were: *Escherichia coli* isolates EC51 (lane A), EC2 (B), EC8 (C), EC50 (D) and K12 (E); *Enterobacter cloacae* isolates MC17 (G) and NJ4 (H); *Serratia rubidea* isolates O13 (I) and O10 (J); *Salmonella arizonae* O8 (K); and *Hafnia alvei* W12 (L).

Table 3. ELISA of mAb 15D8 versus non-motile cells, motile cells and flagellar protein

Bacterium	Strain	A_{414}		
		Non-motile*	Motile†	Flagella
<i>Escherichia coli</i>	EC2	0.06	0.82	0.75
<i>Escherichia coli</i>	EC4	0.03	0.82	0.45
<i>Escherichia coli</i>	EC8	0.05	0.25	0.71
<i>Escherichia coli</i>	EC10	0.07	0.87	1.25
<i>Enterobacter agglomerans</i>	MC14	0.08	0.70	ND
<i>Enterobacter agglomerans</i>	GBP7	0.05	2.45	ND
<i>Enterobacter cloacae</i>	OH12	0.09	0.87	ND
<i>Pseudomonas aeruginosa</i>	CA6	NA	0.11	0.17
<i>Pseudomonas aeruginosa</i>	WS48	NA	0.18	0.16

ND, Not done.

NA, Not applicable (no non-motile isolates were found).

* Microscopic examination of sample wet mounts and the lack of motility on BHI/0.4% agar plates confirmed these strains to be non-motile.

† Motile cells for each strain were induced by three passages of non-motile cells through U-tubes. Fresh preparations of whole bacteria were used to coat the ELISA plates.

Klebsiella and *Pseudomonas* species. These results are not surprising, since *Klebsiella* and *Shigella* species do not possess flagella.

Fig. 3. Immunoblot of enterobacterial flagellin proteins using mAb 15D8. Samples and M_r markers are identical to those in Fig. 2.

Induction of motility

The absence of reactivity to mAb 15D8 in non-motile *Shigella* and *Klebsiella* strains further supports the specificity of the antibody for the bacterial flagella. The inability of 15D8 to react with all the isolates of a genus (Table 2) may be due to the absence of flagella. To test this hypothesis, several isolates which did not give a positive reaction by ELISA were microscopically examined for motile cells. If possible, motile cells were induced and partially purified flagella were prepared. Table 3 shows that for each isolate tested, antibody reactivity closely correlated with cell motility. In each case the antibody did not react with non-motile cells; however, of the organisms which could be induced to motility, good reactivity with the antibody was observed. Accordingly, flagellar proteins isolated from the same strains also reacted well with the antibody. No antibody reactivity was found either with motile cells or with purified flagellin from the *Pseudomonas* isolates examined, thereby supporting our assumption that the 15D8 epitope is present only on motile enterobacterial species.

Heterogeneity of flagella size

Flagella are known to be heterogeneous in size. Flagellin preparations from several enterobacterial species were fractionated by SDS-PAGE, blotted onto nitrocellulose

paper, and probed with mAb 15D8 to determine whether its epitope was present only on some strains of a particular size. Fig. 2 shows the migration patterns of 11 enterobacterial flagellin preparations. Flagellin monomers from different isolates of *Enterobacter* (lanes G and H; M_r 28000) and *Serratia* (lanes I and J; M_r 47–48000) showed only slight variations in apparent M_r as compared to the *Escherichia* isolates (lanes A–E; M_r 36–68000). The immunoblot showed that the flagellin proteins from all the isolates examined reacted strongly with the antibody (Fig. 3). The minor antibody-reactive bands, observed mostly in the *E. coli* samples (Fig. 3, lanes A–E) and as a doublet in the *Enterobacter* samples, are probably degradation products of flagellin. An immunoblot of a cell extract of *Enterobacter* showed only a single antibody reactive protein (Fig. 1, lane A2).

Discussion

SDS-PAGE analyses of flagellar proteins from *E. coli* K12 and six *E. coli* EC strains showed that only a single polypeptide was isolated from each strain but that the M_r of this protein varied greatly. The apparent M_r values of these flagellar proteins confirmed previously reported values for *Serratia*, *E. coli* and *Pseudomonas* (Kondoh & Hotani, 1974; Allison *et al.*, 1985; McDonough & Smith, 1976). Significant variation in flagellin M_r has been observed only for *E. coli* and *Salmonella* species (Homma *et al.*, 1987; Joys, 1988; Langman, 1972). The existence of evolutionary conserved and variable regions on the flagellin structural gene (*hag*) has been proposed by Iino (1977) and other investigators (Lawn, 1977; McDonough, 1965; McDonough & Smith, 1976). Genetic and amino acid analyses have confirmed that the amino- and the carboxy-terminus of flagellin proteins from various genera appear to be highly conserved, whereas the sequences are more variable towards the central regions of flagellin (Joys, 1976, 1985; Kuwajima *et al.*, 1986; Szekely & Simon, 1983; Wei & Joys, 1985). This information correlates with the recent findings that the carboxy-terminus of flagellin is essential for flagellar polymerization and excretion (Homma *et al.*, 1987), and the variable flagellin immunogenicity reflects the central region of the gene (Joys, 1976, 1985; Kuwajima, 1988a). The clustering of essential bases at the terminus of the flagellar gene would allow for mutations within the central region without affecting flagella assembly and function. Kuwajima (1988b) used deletion analysis to confirm that 187 amino acid residues could be deleted from the central region of *E. coli* K12 flagellin without destroying flagella formation or functionality. Mutations within this central, variable region would account for the variations in H-antigen serotypes within a genus (Joys,

1976; Yamaguchi *et al.*, 1984). Variations in H-antigen types have previously been shown to be closely associated with flagellin morphology in *E. coli* (Kuwajima, 1988a; Lawn *et al.*, 1977) and with variations in flagellin M_r in *Pseudomonas aeruginosa* (Allison *et al.*, 1985) and *Campylobacter* (Harris *et al.*, 1987). Insertions and deletions in this central region could also explain the heterogeneity in *E. coli* flagellar protein sizes observed in our study.

A flagella preparation from *E. coli* was used to produce a mAb, 15D8, which reacted with enterobacterial flagella. Previously, Ibrahim *et al.* (1985b, c) found that polyclonal sera produced against *Salmonella* flagellar protein cross-reacts with flagella from *E. coli* and other members of the Enterobacteriaceae. However, the flagellar protein had to be heat-denatured or in the unfolded-state in order to show serological reactivity with the antibody. This suggests that the epitope reported by Ibrahim *et al.* (1985c) is most likely an internal epitope that only becomes accessible to antibody under denaturing conditions. Similar results were obtained in the analysis of *Campylobacter* flagella, where the conserved or broadly cross-reactive epitopes were predominantly internal and only immunoreactive after denaturation (Harris *et al.*, 1987; Logan & Trust, 1986). The results of our studies suggest that the epitope for mAb 15D8 is probably surface exposed and linear, since antibody reactivity to enterobacterial flagella was obtained with either undenatured flagellar protein (ELISA) or with SDS-denatured antigen (immunoblots). One interesting exception however, was a flagella preparation of a laboratory strain of *E. coli* K12, which reacted with 15D8 only on immunoblots. It appears, therefore, that some denaturation of K12 flagellin is essential to expose this epitope for antibody reactivity. The broad reactivity of 15D8 also suggests that the antibody might be useful for the detection of coliforms isolated from environmental or clinical specimens.

The analysis of flagellar antigens with mAb 15D8 under both denaturing and non-denaturing conditions shows that the epitope to this antibody is conserved in the Enterobacteriaceae. All *E. coli* flagellin proteins tested, which varied greatly in M_r , also reacted with the antibody. These results would suggest that the epitope for mAb 15D8 is most likely located on the conserved region of the flagellin protein. Additional supporting evidence on the conserved nature of this epitope was obtained when cell extracts of *Salmonella* were examined. The genetic sequences for phase I and II flagellin in *Salmonella* are homologous but not identical, hence the products are antigenically distinct (Joys, 1988; McDonough, 1965). Immunoblots (unpublished data) of *S. typhimurium* cell extracts showed that mAb 15D8 reacted with two proteins (doublet) with the apparent M_r values

of the phase I and II flagellin types. In addition, the IgA antibody MOPC467, which also reacts with both phase types of *Salmonella* flagella (Smith & Jones, 1983), reacted with the same protein doublet as 15D8 on immunoblots of *S. typhimurium* extracts.

The highly conserved nature of this epitope across M_r , phase variation and generic barriers would suggest that it may be vital to flagella structure and function. Mapping the epitope using site-directed mutagenesis should further define the role and the location of the epitope. mAb 15D8 should be useful in studies of flagellar transport, assembly and polymerization as well as in genetic regulation of flagella morphogenesis.

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