Antigenic diversity in flagellar epitopes among *Bacillus piliformis* isolates

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Summary. Monoclonal antibodies (MAbs) were developed to *Bacillus piliformis* isolate-specific flagellar epitopes and used to group *B. piliformis* isolates on the basis of epitope expression. BALB/c mice immunised with flagella purified from various *B. piliformis* isolates served as the source of immune spleen cells for fusion with SP2/0Ag14 myeloma cells. Evaluation of hybridoma culture medium by ELISA against various bacterial species and *B. piliformis* isolates indicated that 482 of 2127 hybridomas secreted antibodies specific for *B. piliformis*. Specificity of MAbs for flagellar epitopes was demonstrated by indirect fluorescent antibody assays and Western blot analyses. Probing of 10 *B. piliformis* isolates with MAbs indicated that four *B. piliformis* isolates each possessed a distinct and isolate-specific flagellar epitope; five other isolates shared a common flagellar epitope. One isolate did not react with any of the MAbs specific for flagellar epitopes. Thus, *B. piliformis* isolates could be grouped into six antigenically distinct groups based upon flagellar epitope expression. Additionally, a MAb reactive with a cell-associated component recognised all but one isolate. This serological grouping of *B. piliformis* isolates agrees with the grouping of isolates based upon genetic and physiological characteristics, and supports the assertion that there are different strains among *B. piliformis* isolates.

Introduction

Bacillus piliformis, the aetiological agent of Tyzzer's disease, is an unclassified, large, motile, gram-negative, obligately intracellular, spore-forming bacterium. Since the original observation of the organism in mice by Tyzzer in 1917,¹ *B. piliformis* has been identified in the gastrointestinal tract and liver of various laboratory and domestic animals, including mice, rats, hamsters, gerbils, rabbits, rhesus monkeys, dogs and several other species.² Physiological criteria used in the classification of bacteria cannot be used with *B. piliformis* because this organism cannot be propagated on cell-free media.³ Thus, routine classification of an organism as *B. piliformis* is based solely on morphological and pathological characteristics.

Evidence of strain differences among *B. piliformis* isolates was first suggested in the early 1970s by experimental pathogenicity and vaccination studies in mice.^{4–6} Host specificity and protection afforded by vaccination was most complete when the challenge isolate of *B. piliformis* was the same as the isolate used for vaccination. Further evidence of diversity among the various *B. piliformis* isolates was demonstrated by agar gel diffusion, complement fixation tests and

Western blot analyses. These tests revealed that both conserved and isolate-specific antigens were present in the various *B. piliformis* isolates.^{4,7} Subsequent studies by Motzel and Riley⁸ suggested that *B. piliformis* flagella expressed both conserved and isolate-specific epitopes.

Further evidence of antigenic diversity among *B. piliformis* isolates was provided by Toriumi *et al.*⁹ who demonstrated that a monoclonal antibody (MAb), developed to a flagellar epitope of a *B. piliformis* isolate of rat origin, reacted with only three of seven *B. piliformis* isolates. In addition to the antigenic variability demonstrated by the above studies, investigations by Riley *et al.*⁷ demonstrated heterogeneity in morphological and physiological characteristics among 10 *B. piliformis* isolates.

The studies cited above indicated that isolates of *B. piliformis* represent a diverse group of organisms. Furthermore, these studies also suggested that *B. piliformis* isolates might be grouped antigenically on the basis of expression of isolate-specific flagellar epitopes. In this study, we report the development of MAbs reactive with isolate-specific flagellar epitopes and grouping of *B. piliformis* isolates based upon reactivity with these MAbs.

Materials and methods

Reagents and equipment

Reagents and equipment used in this study were as follows: Dulbecco's Modified Eagle's Medium and polyethylene glycol 4000 (Sigma); Omnimixer homogeniser (Omni International, Waterbury, CT, USA); HL-1 Ventrex synthetic medium (Endotronics, Coon Rapids, MN, USA); Falcon 3912 assay plates (Becton Dickinson, Oxnard, CA, USA); peroxidase-labelled goat anti-mouse IgG and FITC-labelled donkey anti-mouse IgG (Jackson Immuno Research Labs, West Grove, PA, USA); ABTS, peroxidase-labelled goat anti-mouse IgA and tetramethyl benzidine substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA); peroxidase-labelled goat anti-mouse IgM (Zymed, San Francisco, CA, USA); peroxidase-labelled goat anti-mouse IgG1, IgG2a, IgG2b and IgG3 (Fisher Biotech, Pittsburgh, PA, USA); mol. wt standards (BioRad Laboratories, Richmond, CA, USA); biotinylated horse anti-mouse IgG and peroxidase-labelled streptavidin (Vector Laboratories, Burlingame, CA, USA).

Bacterial strains

Ten *B. piliformis* isolates of equine (E), guinea pig (GP), rabbit (B), mouse (M), hamster (H1, H2 and H3), gerbil (G), and rat (R1 and R2) origin were used in the study. The isolation and origin of isolates have been described previously.⁷

Animals

Female VAH BALB/c mice, 6–8 weeks old, free of clinical disease and infection with known pathogenic organisms, were obtained from the National Cancer Institute. Gerbils known to be free of infection with *B. piliformis* were obtained from a breeding colony at the University of Missouri. All experiments were conducted under an approved Animal Care and Use Protocol and animals were maintained in compliance with PHS policy.

Antigen preparation

B. piliformis isolates were grown and harvested as described by Riley *et al.*⁷ Briefly, *B. piliformis* were propagated in established Buffalo rat liver (BRL) or mouse fibroblast (3T3) mammalian cell lines cultured in Dulbecco's Modified Eagle's Medium supplemented with fetal calf serum 5–10% and 2 mM L-glutamine at 37°C with CO₂ 10%. Infected cell monolayers were harvested, sonicated to release intracellular bacteria, centrifuged at 2000 *g* for 10 min at 4°C to pellet the mammalian cells, and the supernate was centrifuged at 10000 *g* for 30 min at 4°C to pellet *B. piliformis*. After washing once with PBS, pH 7.4, *B. piliformis* organisms were used as antigen in ELISA, IFA and Western blot analyses. Isolated flagella were prepared as described previously⁸ and used as immunogen for MAb

development. Briefly, *B. piliformis* flagella were sheared in a homogeniser (Omnimixer) at speed 5 for 60 s and centrifuged at 12000 *g* for 30 min at 4°C to pellet intact organisms. Flagella were pelleted by centrifugation twice at 58000 *g* for 2 h at 4°C, and resuspended in sterile PBS, pH 7.4. Protein concentrations were determined by the BCA method with bovine serum albumin as standards.

Development of MAbs

MAbs were produced by the procedure of St Groth and Scheidegger¹⁰ as previously described.¹¹ Three female VAH BALB/c mice, 6–8 weeks old, were immunised by intravenous injection with 10 µg of isolated flagella and used in three separate fusion experiments. One BALB/c mouse was inoculated with *B. piliformis* flagella pooled from isolates R1, B and E. A second mouse was inoculated with flagella pooled from isolates M, GP, H1 and G, and a third mouse was inoculated with flagella from isolate M. At 4–6 weeks after primary injection, each mouse was given, by intravenous injection, 10 µg of the same flagellar proteins as those used for primary immunisation. Spleens from immunised mice killed 4 days after the secondary injection served as the source of immune cells for hybridisation experiments.

Spleen cells were prepared aseptically by teasing the donor spleen in serum-free HL-1 Ventrex medium supplemented with 2 mM L-glutamate, penicillin and streptomycin (HL-1 medium). For fusion, 1.5×10^8 spleen cells were fused with 5×10^7 viable SP2/0Ag14 mouse myeloma cells grown in HL-1 medium; polyethylene glycol 4000, 50% in HL-1 medium, was used as the fusing agent. Fused cells in HL-1 medium supplemented with fetal calf serum 2%, hypoxanthine, aminopterin and thymidine (HAT medium), were distributed into the wells of 15 96-well culture plates seeded 24 h earlier with 1000 BALB/c peritoneal macrophages/well in HAT medium. Cultures were fed at 7 days with 150 µl of HAT medium; 10–20 days after fusion, medium from wells containing vigorously growing hybridomas were screened by ELISA as described below.

ELISA

The procedure used in this study followed the methodology described by our laboratories for evaluation of MAbs to *Campylobacter* spp.¹¹ Initial screening of hybridoma culture medium was for reactivity with pooled isolates of *B. piliformis* air-dried on to Falcon 3912 assay plates at 1.5×10^7 organisms/well; spent SP2/0 culture medium served as negative control medium. Reactivity was detected with peroxidase-labelled, goat anti-mouse IgG (Fc-specific) diluted 1 in 5000 in blocking buffer as secondary antibody, and ABTS as chromophore. Hybridomas producing OD readings of > 0.20 were considered to be positive reactors.

Hybridomas demonstrating reactivity with *B. pili-*

formis were also tested by ELISA for reactivity with BRL cells, 3T3 cells and a mixture of *Pseudomonas aeruginosa*, *Proteus vulgaris* and *B. cereus*. Hybridomas demonstrating readings of < 0.03 OD were considered to be negative reactors with these antigens. Hybridomas that demonstrated specificity for *B. piliformis* were expanded into 24-well culture plates, tested again for specificity, cloned by limiting dilution, and again tested for specificity.

Isotyping of MAbs

B. piliformis-sensitised assay plates were used to isotype MAbs as described previously.¹² After incubation of hybridoma supernate with antigen-coated wells, bound antibodies were detected by incubation with peroxidase-labelled goat anti-mouse IgA, IgM, IgG1, IgG2a, IgG2b and IgG3. Incubations, colour development and plate reading were as described above.

Indirect immunofluorescent antibody assay (IFA)

Twelve-well glass slides were loaded (10 µl/well) with individual *B. piliformis* isolates at c. 10⁶ cells/ml, air-dried at room temperature and fixed in cold acetone for 10 min. Hybridoma supernate diluted in PBS, pH 7.4, containing Tween 20 0.05% and powdered non-fat dried milk 2.5% (blocking buffer) were added to individual wells and incubated for 30 min at room temperature in a humidified chamber. After three 5-min washes with PBS, pH 7.4, containing Tween 20 0.05% (wash buffer), FITC-labelled, donkey anti-mouse IgG (Fc-specific) diluted 1 in 80 in wash buffer was added to each well and incubated for 30 min at room temperature in a humidified chamber. After four 5-min washes with wash buffer and two 5-min washes with PBS, pH 7.4, the slides were mounted and examined for specific fluorescence. Negative controls consisted of *B. piliformis* incubated with spent SP2/0 medium and MAbs incubated with a mixture of *P. aeruginosa*, *Pr. vulgaris* and *B. cereus*.

Indirect immunofluorescent antibody assays were also performed on *B. piliformis*-infected BRL cells and specimens of caecum and liver collected from gerbils 5 days after oral infection with 10⁸ viable *B. piliformis* organisms (R1 isolate). Infected BRL cells and formalin-fixed tissues embedded in paraffin, cut to 2 µm sections, mounted on glass slides, and rehydrated with PBS were processed for IFA staining as described above. Non-infected gerbil caecum and liver and BRL cells incubated with spent SP2/0 medium served as negative controls. Sections (5 µm) adjacent to the IFA stained sections were examined after Warthin-Starry staining to confirm specificity of the MAbs for *B. piliformis*.

SDS-PAGE

To identify individual peptides and glycoproteins recognised by the MAbs, whole *B. piliformis* organisms were subjected to SDS-PAGE under reducing con-

ditions on vertical slab gels, as described by Laemmli.¹² Proteins were stacked with acrylamide 4.5% w/v and separated on acrylamide 8% w/v. Mol. wt standards consisted of rabbit muscle phosphorylase B, bovine serum albumin, hen egg white ovalbumin, bovine carbonic anhydrase B, soybean trypsin inhibitor and hen egg white lysozyme.

Immunoblotting

Components of *B. piliformis* separated by SDS-PAGE were transferred on to nitrocellulose membranes by the method of Towbin.¹³ After membranes were blocked overnight at room temperature by incubation in blocking buffer, they were probed for reactivity with the MAbs and developed with biotinylated goat anti-mouse IgG and peroxidase-labelled streptavidin as described previously.⁷ Antibody reactive bands were visualised by incubation with tetramethyl benzidine substrate.

Results

Monoclonal antibody reactivity

During the course of three separate hybridisation experiments, 482 of 2127 hybridomas demonstrated reactivity with *B. piliformis* isolates; < 10% (32/482) of the MAbs reactive with *B. piliformis* demonstrated reactivity with *P. aeruginosa*, *Pr. vulgaris*, *B. cereus*, BRL cells or 3T3 cells. Based upon preliminary studies, 24 hybridomas were selected for more extensive characterisation. Specificity testing of these MAbs by ELISA demonstrated no reactivity with various bacteria that included: *Citrobacter freundii*, *Klebsiella pneumoniae*, *Acinetobacter calcoaceticus*, *Enterobacter aerogenes*, *Salmonella typhimurium*, *K. marcescens*, *Streptococcus faecalis*, *Staphylococcus epidermidis*, *Campylobacter jejuni*, *Pr. vulgaris*, *Escherichia coli*, *Vibrio fluvialis*, *Shigella sonnei* and *Ent. cloacae*.

MAbs specific for *B. piliformis* demonstrated various reactivity patterns when tested by ELISA with the 10 *B. piliformis* isolates (table). MAbs with single isolate specificity were demonstrated for isolates B, E, R1 and M. Hybridomas secreting antibodies that recognised epitopes on more than one isolate exhibited two reactivity patterns. One group of MAbs reacted exclusively with isolates GP, H1, H2, H3 and G; a second group of MAbs reacted with all *B. piliformis* isolates except B and a high culture passage of R1. Isolate R2 was unique in that it reacted only with the latter group of MAbs.

Isotyping

The selected MAbs were further characterised by analyses for antibody isotype. All MAbs were of the IgG1, IgG2a or IgG2b isotype (table).

Indirect fluorescent antibody assays (IFA)

MAbs demonstrating specificity for *B. piliformis* by

Table. Characterisation of MAbs

MAb	Isolate specificity	Isotype	Epitope location	Antigen size (kDa)
903-13C1	R1	IgG1	Flagella	54
903-13D3	E	IgG2a	Flagella	54
903-7C12	B	IgG2b	Flagella	54
916-6A9	M	IgG2a	Flagella	54
911-8C5	G, GP, H1, H2, H3	IgG1	Flagella	54
903-12G9	R1, R2, E, M, G GP, H1, H2, H3†	IgG1	Cell-associated	*

* No definitive peptide identified.

† Not reactive with isolate B and a late culture passage of R1.

Fig. 1. IFA stains of *B. piliformis* isolate R1: A, with MAb 903-13C1 demonstrating specificity for flagella—the photograph shows the bacteria as a dark non-staining rod surrounded by fluorescein-labelled peritrichous flagella (bar, 5 μ m); B, within an infected BRL cell probed with MAb 903-13C1—*B. piliformis* organisms show as dark non-staining rods (arrow) surrounded by clumped, fluorescein-labelled flagella (bar, 8 μ m).

ELISA were tested by IFA to determine the structural location of the epitope recognised and to confirm specificity of the antibodies. MAbs specific for *B. piliformis* by ELISA demonstrated identical isolate and group specificities by IFA. Furthermore, IFA assays confirmed that antibodies did not react with other bacteria or with uninfected BRL and 3T3 cells. IFA tests also demonstrated that most of the *B. piliformis*-specific MAbs reacted with epitopes located on flagella. Fig. 1A demonstrates the reactivity of an R1-specific MAb, with flagella of *B. piliformis* isolate R1 from culture; MAbs reactive with other isolates demonstrated the same flagellar specificity (table). MAbs that demonstrated ELISA reactivity with all *B.*

Fig. 2. Western blot of *B. piliformis* isolates B, E, H1, GP, G, M and R1 probed with MAb 911-8C5. Note the primary band at c. 54 kDa defined with isolates GP, H1 and G. Lower mol. wt bands are believed to be break-down products of the 54-kDa protein. The approximate migration of mol. wt markers is indicated.

piliformis isolates except B and the late culture passage of R1 reacted with a cell-wall-associated epitope and showed no reactivity with flagella. Isolate R2 did not demonstrate any flagellar reactivity and reacted only with MAbs reactive with the cell-wall-associated epitope.

To confirm that the MAbs would recognise intracellular *B. piliformis*, and that the flagellar epitope(s) recognised was not a culture-induced antigen, MAbs specific for the R1 isolate were evaluated by IFA with *B. piliformis*-infected BRL cells and caecum and liver tissue sections from gerbils infected experimentally with the R1 isolate of *B. piliformis*. MAbs specific for the R1 isolate of *B. piliformis* demonstrated strong reactivity with isolate R1 flagella in both infected BRL cells (fig. 1B) and infected gerbil tissues (not shown). R1-specific MAbs did not show any specific fluorescence when tested by IFA with uninfected gerbil and liver tissue sections. MAbs specific for other *B. piliformis* isolates did not show any reactivity with the R1 isolate in tissue sections.

Immunoblotting

Immunoblots of *B. piliformis* components separated by SDS-PAGE and probed with the *B. piliformis*-specific antibodies confirmed the isolate and group specificity of the MAbs. MAbs that demonstrated flagellar reactivity by IFA reacted with epitopes

located on a protein of *c.* 54 kDa. When a MAb specific for isolates GP, H1, H2, H3 and G was used to probe Western blots of different *B. piliformis* isolates, specific immunoreactive bands were observed with isolates G, H1 and GP, and were not present with isolates M, R1, E or B (fig. 2). Other MAbs that showed flagellar specificity by IFA also reacted with a protein of *c.* 54 kDa and confirmed specificities demonstrated by ELISA and IFA (table). Immunoblots probed with MAbs reactive with the *B. piliformis* cell-wall-associated epitope failed to develop any definitive band.

Discussion

In the present study, MAbs reactive with flagellar epitopes of *B. piliformis* were developed to nine of 10 isolates; none of the flagella-specific MAbs reacted with isolate R2, of rat origin. The specificity of the MAbs for *B. piliformis* was demonstrated by a lack of reactivity in ELISA against a wide variety of other bacterial species. Furthermore, specificity of the MAbs for flagellar epitopes of *B. piliformis* was demonstrated by IFA and Western blotting. IFA staining of liver and intestinal tissue sections from gerbils infected experimentally with isolate R1 further confirmed the specificity of the MAbs and demonstrated that the flagellar epitopes recognised by the antibodies were not culture-induced epitopes.

Based upon our results, the isolates could be grouped into six serological groups with the flagella-specific MAbs. In this grouping, isolates, B, E, M and R1 each possess a distinct flagellar epitope and represent four distinct serological groups. Isolates G, GP, H1, H2 and H3 share a common flagellar epitope and represent a fifth serological group. Isolate R2 does not possess a flagellar epitope recognisable by any of our flagella-specific MAbs and represents the sixth serological group. These results are consistent with previous reports that suggested considerable heterogeneity among *B. piliformis* isolates.^{4-6, 8, 9}

The grouping of *B. piliformis* isolates on the basis of expression of flagellar epitopes is in agreement with observations made in our laboratories on the growth and physiological characteristics of the isolates. For example, isolates GP, H1, H2, H3 and G, which demonstrate similar growth and sporulation rates in culture,⁷ are grouped into the same antigenic group by

MAb 911-8C5. Also, at least three of the isolates in this group (GP, H1 and G) produce similar, if not identical, toxins.¹⁴ The observation that isolate R2 did not react with any of the MAbs specific for flagellar epitopes of the other *B. piliformis* isolates is also consistent with previous observations. The growth and physiological characteristics of this isolate differ from those of the other isolates, in that isolate R2 is difficult to grow in the vegetative phase and shows a much higher degree of sporulation than do other isolates (unpublished data). These data suggest that at least one additional serological and physiological group is present in *B. piliformis* and is represented by isolate R2.

The observation that *B. piliformis* isolates express isolate-specific flagellar epitopes was not surprising, since previous studies suggested that *B. piliformis* possesses both conserved and isolate-specific antigens.^{4, 7, 8} However, the failure to develop a MAb that reacted with a flagellar epitope common to all *B. piliformis* isolates was unexpected. A previous study by Toriumi *et al.*⁹ demonstrated that a MAb developed to an isolate of rat origin also reacted with an isolate of mouse origin and suggested the presence of a conserved flagellar epitope. A conserved flagellar epitope in *B. piliformis* was also suggested by the study of Motzel and Riley.⁸ One MAb developed in this current study reacted with all *B. piliformis* isolates except isolate B. However, IFA and Western blot analyses indicated that the epitope recognised by this antibody was associated with the cell and not with flagella. Our data also indicated that this MAb reacted with early culture passages of isolate R1 but failed to react with a late culture passage of R1. These data suggest that long-term culture *in vitro* of at least some *B. piliformis* isolates may result in the loss of this cell-wall-associated antigen.

Although bacterial flagella are best known for their role in motility, they also play an important role in pathogenesis and may function as virulence factors.¹⁵⁻¹⁸ Current studies in our laboratories are directed towards determining the relationship(s) among flagellar epitopes (serological groups), pathogenesis and host specificity in experimental and naturally-occurring *B. piliformis* infections.

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