Identification of the Outer Membrane Proteins of *Campylobacter pyloridis* and Antigenic Cross-reactivity between *C. pyloridis* and *C. jejuni*

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The outer membrane and surface exposed proteins of four strains of the gastric *Campylobacter*-like organism *Campylobacter pyloridis* were identified by SDS-PAGE of Sarkosyl-insoluble membranous material and ^{125}I -surface-labelled whole bacteria. Although constant outer membrane proteins (molecular mass 61, 54 and 31 kDa) were observed in these strains, several variable ^{125}I -labelled surface proteins were detected. *C. pyloridis* does not appear to express a single surface-exposed major outer membrane protein like that of *C. jejuni* and *C. coli*. Putative flagella proteins were identified from isolated flagella and acid-extractable surface material and by immunoblotting with anti-flagella antibodies. Several major protein antigens were observed by immunoblotting with anti-*C. pyloridis* antisera. At least two of these antigens cross-reacted with anti-*C. jejuni* antiserum. This cross-reaction appears to be caused primarily by flagellar antigens. However, one major protein antigen (61 kDa) was not cross-reactive with *C. jejuni* and may, therefore, be useful in serological tests for the specific diagnosis of *C. pyloridis* infections.

INTRODUCTION

Campylobacter pyloridis is a recently recognized microaerophilic, S-shaped bacterium (Warren & Marshall, 1983), previously termed gastric *Campylobacter*-like organism type 1 (GCLO-1) which colonizes the gastric mucosa of patients with active, chronic gastritis and peptic ulceration (McNulty & Watson, 1984; Langenberg *et al.*, 1984; Booth *et al.*, 1986; Jones *et al.*, 1984). Despite this close association with abnormal gastric pathology the pathogenic, as distinct from opportunistic, nature of this organism has yet to be proven. Nevertheless, the presence of *C. pyloridis* may be an important consideration in the treatment of gastric disease.

Patients colonized with *C. pyloridis* elicit a specific antibody response (Jones *et al.*, 1984; Kaldor *et al.*, 1985) potentially useful as a diagnostic aid and for monitoring the disease state during treatment. Consequently ELISA systems are being developed to detect serum anti-*C. pyloridis* antibodies (Booth *et al.*, 1986). However, preliminary studies suggest that *C. pyloridis* displays antigenic cross-reactivity with the thermophilic campylobacters *C. jejuni* and *C. coli* (Hutchinson *et al.*, 1985; Newell, 1986*b*), which could result in lack of specificity with whole cell antigens.

The aim of these investigations was to identify the outer membrane and surface proteins, including flagella, of *C. pyloridis* and to establish which of these proteins were antigenically cross-reactive with the surface antigens of *C. jejuni*.

METHODS

Bacterial strains. *C. pyloridis* strains NCTC 11637 and NCTC 11638 were isolated by Dr B. Marshall (Royal Perth Hospital, Perth, Australia) and kindly supplied by Dr M. B. Skirrow (Worcester Royal Infirmary, Worcester, UK). *C. jejuni* strain 81116 was described by Newell *et al.* (1984). All other strains were obtained from gastric biopsies and were isolated on blood agar (blood agar base no. 2; Oxoid) containing 5% (v/v) defibrinated horse blood and 2% (w/v) agar with or without Skirrow's antibiotics (Skirrow, 1977) in microaerophilic conditions and stored in 10% (w/v) glycerol in 1% (w/v) proteose peptone in liquid nitrogen.

Antisera and antibodies. Rabbit antisera were prepared against *C. pyloridis* strain 11637, *C. jejuni* strain 81116 and *C. jejuni* flagella (Newell *et al.*, 1984). The production and characterization of the monoclonal antibody CF5 was described by Newell (1986a, b).

Outer membrane preparation. Outer membranes of *C. pyloridis* strains NCTC 11637, NCTC 11638, 85033 and 85034 were prepared by solubilization of crude membrane preparations with sodium *N*-lauroyl sarcosinate (Newell *et al.*, 1984).

Acid extraction. Acid extracts of whole organisms were prepared from strains NCTC 11637, NCTC 11638 and 85033 by incubation in 0.2 M-glycine/HCl buffer, pH 2.2 (Newell *et al.*, 1984).

Flagella preparation. *C. pyloridis* strain 85033 was cultured in a broth containing 1% (w/v) peptone, 1% (w/v) tryptose, 0.1% (w/v) dextrose, 0.2% (w/v) yeast extract (all from Oxoid), 0.5% (w/v) sodium chloride, 0.01% (w/v) sodium metabisulphite, 10% (v/v) horse blood lysate and Skirrow's antibiotics at 37 °C in microaerophilic conditions and rotated on an orbital shaker at 80 r.p.m. Flagella were sheared from the bacteria and purified by differential centrifugation (Newell *et al.*, 1984). The preparation was monitored by negative staining and transmission electron microscopy, and by SDS-PAGE.

¹²⁵I-surface-labelling of whole bacteria. *C. pyloridis* strains were harvested from blood agar plates and washed twice by centrifugation (5000 g, 10 min, 4 °C) in phosphate-buffered saline (PBS: 140 mM-sodium chloride, 2.7 mM-potassium chloride, 8 mM-disodium hydrogen phosphate and 1.5 mM-potassium dihydrogen phosphate, pH 7.2). Bacteria were ¹²⁵I-surface-labelled using 1,3,4,6-tetrachloro-3 α -6 α -diphenyl-glycouril (Iodogen) as described by Newell *et al.* (1984). The specific activity of ¹²⁵I-labelled bacteria was approximately 1 μ Ci (μ g protein)⁻¹ [37 kBq (μ g protein)⁻¹].

SDS-PAGE. A 10–25% (w/v) linear gradient SDS-PAGE system was used (Lambden *et al.*, 1979). Protein bands were stained with Kenacid blue (BDH). The protein molecular mass markers (Sigma) were α -lactalbumin, trypsin inhibitor, trypsinogen, carbonic anhydrase, glyceraldehyde-3-phosphate dehydrogenase, egg albumin and bovine albumin.

Electroimmunoblotting. SDS-PAGE gels of the total proteins, outer membrane preparations, acid extracts and flagella preparations were electroblotted onto Hybond paper (Amersham) at 55 V for 18 h at 4 °C in 24 mM-Tris, 190 mM-glycine and 20% (v/v) methanol, pH 8.0. Non-specific binding was blocked by incubation with 3% (w/v) gelatin in 10 mM-Tris/HCl, 150 mM-sodium chloride containing 0.05% (v/v) Tween 20 (TTBS), pH 7.4, for 2 h at 20 °C. The blots were incubated in rabbit antiserum (1 in 100) diluted in 50 mM-Tris/HCl buffer, pH 7.4, containing 150 mM-sodium chloride, 5 mM-EDTA, 0.25% (w/v) gelatin and 0.05% (v/v) Nonidet P-40 (incubation buffer) or in undiluted hybridoma supernatant fluid containing 20 mM-HEPES (pH 7.4) for 2 h at 20 °C. After washing in TTBS the blots were incubated in goat anti-rabbit IgG (diluted 1 in 3000 in incubation buffer) (BioRad) for 1 h at 20 °C or in ¹²⁵I-labelled sheep anti-mouse IgG (diluted to 1 $\times$ 10⁶ c.p.m. ml⁻¹) in incubation buffer [Amersham; specific activity 5–20 μ Ci μ g⁻¹ (185–740 kBq μ g⁻¹)] overnight at 4 °C. The unbound material was removed by extensive washing in TTBS. Bound peroxidase activity was detected using 4-chloro-1-naphthol and hydrogen peroxide. Bound ¹²⁵I-labelled material was detected by autoradiography.

ELISA. Antigen [10 μ g protein (ml whole cell sonicate)⁻¹ or 5 μ g protein (ml acid extract)⁻¹] in 100 μ l 0.1 M-potassium carbonate buffer, pH 9.6, was adsorbed onto microELISA plates (Dynatech) overnight at 20 °C. The wells were washed in ELISA wash (145 mM-sodium chloride containing 0.05% v/v, Tween 20) and incubated with appropriate dilutions of rabbit antisera for 2 h at 37 °C. After washing, the wells were incubated with 100 μ l goat anti-rabbit IgG coupled to peroxidase (Miles Research Labs) (1 in 1000 dilution in ELISA wash containing 1% w/v, bovine serum albumin and 5 mM-Tris/HCl, pH 7.6) for 2 h at 37 °C. Bound antibody was detected with 100 μ l tetramethylbenzidine substrate for 15 min, stopped with 50 μ l 1 M-sulphuric acid and read at 450 nm in a microELISA reader (Dynatech). The ELISA titres were calculated as described by Virji *et al.* (1983).

RESULTS

Outer membrane and surface proteins

The total protein profiles of the four strains of *C. pyloridis* were very similar but no significant major protein bands were observed. SDS-PAGE gels of the total protein profile, crude membrane preparation, Sarkosyl-soluble material and Sarkosyl-insoluble membranous material from strain 85033 are shown in Fig. 1a. Three outer membrane proteins (61, 54 and 31 kDa) were seen in this strain. The protein profiles of outer membrane preparations from the other strains showed proteins bands of the same molecular mass though some quantitative differences were observed. The outer membrane preparation, when viewed by transmission electron microscopy, contained membranous material and many 'doughnut-shaped' 11 nm particles. Flagella fragments were rarely seen (Fig. 2a).

Fig. 1. (a) SDS-PAGE gel of *C. pyloridis* strain 85033. Track 1, total protein profile; track 2, crude membrane preparation; track 3, Sarkosyl-soluble membranous material; track 4, Sarkosyl-insoluble outer membrane; track 5, acid extract; and track 6, flagella preparation. All preparations were loaded at 10 µg protein per track and the gel was stained with Kenacid blue. (b) Autoradiograph of SDS-PAGE gel of ^{125}I -surface-labelled *C. pyloridis* strains. Track 1, strain 11637; track 2, strain 11638; track 3, strain 85033; and track 4, strain 85034.

The 61 and 54 kDa proteins, and a 73 kDa major protein, were also present in the flagella preparation (Fig. 1a, track 6). An additional 40–50 kDa protein always ran as a distorted band in this preparation. Untreated organisms expressed multiple flagella many of which were sheathed and had terminal ‘paddles’, which appeared to be extensions of the flagella sheath. Most flagella fragments recovered after shearing were characterized by amorphous terminal blebs at both ends (Fig. 2c). This amorphous material represented a large proportion of the final flagella preparation. Conversely the ‘doughnut-shaped’ particles seen in the outer membranes were infrequently observed in this preparation.

Acid-extracted whole bacteria were without flagella but otherwise appeared intact. Acid extracts from all strains had similar protein profiles and had four major (61, 56, 31 and 25 kDa) proteins (Fig. 1a, track 5).

^{125}I -surface-labelled bacteria (Fig. 1b) demonstrated several variable proteins, including a 71.5–75 kDa protein and a 48–50 kDa protein. A 65.5 and 64.5 kDa doublet band was seen in strains 85033 and 11638; the high molecular mass band only was seen in strain 85034 whilst the lower molecular mass band only was seen in strain 11637. Several ^{125}I -labelled major proteins (60, 31, 26 and 19 kDa) were also observed, which had the same molecular mass in each strain.

Surface antigens of *C. pyloridis*

Rabbit anti-*C. pyloridis* antiserum labelled five major antigens (61, 56, 54, 29 and 26 kDa) in all four strains of *C. pyloridis* (Fig. 3a). Several variable antigens were also observed, including a 26 kDa antigen which was missing in strain 11674. The 61 kDa antigen was a major antigenic

Fig. 2. Transmission electron micrograph of negatively stained (a) outer membrane material showing 11 nm 'doughnut-shaped' particles (bar 0.1 μm), and (b) flagella fragment after mechanical dissociation showing the dispersion of amorphous material from one end (bar 0.1 μm).

Fig. 3. Electroimmunoblots of SDS-PAGE gels of the following: (a) total protein profiles of *C. pyloridis* strain 11637 (track 1), strain 11638 (track 2), strain 85033 (track 3) and strain 85034 (track 4), all incubated with rabbit anti-*C. pyloridis* antiserum. (b) Total protein profile (track 1), outer membrane preparation (track 2), acid extract (track 3) and flagella preparation (track 4) of strain 85033, all incubated with rabbit anti-*C. pyloridis* antiserum. (c) As (a) but incubated with rabbit anti-*C. jejuni* antiserum. (d) As (a) but incubated with rabbit anti-*C. jejuni* flagella antiserum. (e) Total protein profile of *C. jejuni* strain 81116 incubated with rabbit anti-*C. pyloridis* antiserum (track 1) and rabbit anti-*C. jejuni* antiserum (track 2).

component of the outer membrane preparation, while the 56 kDa antigen was a major constituent of the acid extract and flagella preparations. The immunolabelling pattern of this 56 kDa protein indicated that several antigens of similar molecular masses might be present. The 54 kDa antigen was found in the outer membrane and flagella preparations (Fig. 3b).

Cross-reacting surface antigens of *C. pyloridis* and *C. jejuni*

Both *C. jejuni* and *C. pyloridis* sonicated whole cell antigen preparations cross-reacted with the heterologous antisera in ELISA (Table 1). However, the cross-reactivity was greatly reduced when acid extracts of *C. pyloridis* were used. By immunoblotting it could be seen that the major antigens of *C. pyloridis* cross-reacting with rabbit anti-*C. jejuni* were 56 and 54 kDa proteins (Fig. 3c). Rabbit anti-*C. jejuni* flagella antiserum (Fig. 3d) and the monoclonal antibody CF5 labelled the same antigens.

Rabbit anti-*C. pyloridis* antisera labelled a 62 kDa and a diffuse 18 kDa band in blots of whole cell protein profiles of *C. jejuni* 81116 (Fig. 3e). The 62 kDa antigen was also labelled in acid extracts of 81116 and was the major antigen labelled by the homologous antisera. The 18 kDa diffuse band was not detected by anti-*C. jejuni* antisera.

Table 1. *Antigenic cross-reactivity between C. pyloridis and C. jejuni*

Antisera from rabbits, immunized with either *C. pyloridis* or *C. jejuni*, were reacted in ELISA with either whole cell sonicates or acid-extractable material from *C. pyloridis* or *C. jejuni*. The titre was calculated as the dilution of sera which gave an increase in A_{450} of 0.1 after 15 min incubation with the substrate.

Antigen		10 ¹ × ELISA titre		Normal rabbit serum
		Anti- <i>C. pyloridis</i> strain 11637	Anti- <i>C. jejuni</i> strain 81116	
<i>C. pyloridis</i> strain 11637	Whole-cell sonicate	1782	111	0
	Acid extract	111	2	0
<i>C. jejuni</i> strain 81116	Whole-cell sonicate	74	1024	0
	Acid extract	60	270	0

DISCUSSION

The outer membrane and surface exposed proteins of *C. pyloridis* have been identified by ¹²⁵I-surface-labelling and SDS-PAGE of outer membrane material. These techniques have been previously used to identify the outer membrane and surface proteins of other members of the genus *Campylobacter* (Logan & Trust, 1982; Blaser *et al.*, 1983; Newell *et al.*, 1984). The total protein profiles of the *C. pyloridis* strains were similar to those described by Megraud *et al.* (1985). No major outer membrane protein was observed in *C. pyloridis*. Similarly no major surface protein was ¹²⁵I-labelled. Many of the surface exposed proteins that were ¹²⁵I-labelled appear to be peripheral proteins rather than constituents of the outer membrane. Nevertheless the 61 and 31 kDa outer membrane proteins appear to be surface exposed. The thermophilic campylobacters *C. jejuni* and *C. coli* are characterized by a single, 42–47 kDa, surface-exposed outer membrane protein whilst *C. fetus* contains two distinctive outer membrane proteins (Logan & Trust, 1982; Blaser *et al.*, 1983; Newell *et al.*, 1984). It appears that *C. pyloridis* does not possess a major outer membrane protein comparable with those of other campylobacters. The inclusion of GCLO-1 in the genus *Campylobacter* (Anon., 1985) has been questioned (Jones *et al.*, 1985; Goodwin *et al.*, 1985, 1986) on the basis of significant differences in morphology, fatty acids and antibiotic susceptibility. However, other properties of the organism (Wait & Hudson, 1985; Marshall *et al.*, 1984; Megraud *et al.*, 1985) are consistent with the definition of *Campylobacter* (Smibert, 1978).

In previous studies, the 62 kDa flagella protein of *C. jejuni* has been identified after mechanical isolation of flagella. Moreover, this protein was a major component of both outer membrane preparations and acid-extractable surface material. The use of similar techniques did not allow unequivocal identification of the flagella proteins of *C. pyloridis*, the flagella preparation of which gave a complex protein profile, including one protein which appeared to be disturbed during electrophoresis. Such a perturbation may result from changes in ionic strength within the gel, though such effects were not seen with other preparations or from association with non-proteinaceous materials like membrane lipids. The amorphous material, representing much of the flagella preparation, appeared to originate from the terminal blebs of sheathed flagella. Conversely the flagella of the thermophilic campylobacters which remain intact during mechanical dissociation from the cells and fragments of flagella are major components of the outer membrane material from these *Campylobacter* spp. (Newell *et al.*, 1984). Morphological studies by Goodwin *et al.* (1985) suggest that the flagella sheath of *C. pyloridis* is an extension of the outer membrane. The protein profiles of the outer membrane and flagella preparations are not inconsistent with this proposal but the 'doughnut-like' particles, associated with the cell surface (Jones *et al.*, 1985), were infrequent in the flagella preparations. Obviously the flagella of *C. pyloridis* are both morphologically (Jones *et al.*, 1985; Goodwin *et al.*, 1985) and chemically distinct from those of the other campylobacters. Nevertheless, there is considerable antigenic cross-reactivity between the flagella of *Campylobacter* spp. Anti-*C. jejuni* flagella antiserum immunolabelled at least two proteins found in the flagella preparation and acid extract of *C. pyloridis*. Moreover, the monoclonal antibody CF5, which is directed against

the 62 kDa flagella protein of *C. jejuni* (Newell, 1986a) and which cross-reacts with most *Campylobacter* species (Newell, 1986b) also labels these proteins.

The 61 kDa protein was one of the major *C. pyloridis* antigens detected by rabbit anti-*C. pyloridis* antisera which did not cross-react with anti-*C. jejuni* antiserum. Preliminary evidence suggests that a 60–62 kDa protein is a major antigen detectable by electroimmunoblotting and radioimmunoprecipitation using sera from patients colonized by *C. pyloridis* (Newell, 1985). This 61 kDa protein is therefore a candidate antigen for enhancing the specificity and sensitivity of ELISA techniques. The 61 kDa protein is enriched in the acid-extractable material from *C. pyloridis* and has a significantly lower cross-reactivity with rabbit anti-*C. jejuni* antiserum than with whole cell sonicates. Further ELISA investigations using the acid-extractable material for the detection of specific anti-*C. pyloridis* antibody responses in patients with gastritis are in progress.

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