Comparison of Methods Used to Separate the Inner and Outer Membranes of Cell Envelopes of *Campylobacter* spp.

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The outer membrane of *Campylobacter coli*, *C. jejuni* and *C. fetus* cell envelopes appeared as three fractions after sucrose gradient centrifugation. Each outer membrane fraction was contaminated with succinate dehydrogenase activity from the cytoplasmic membrane fraction. Similarly the inner membrane fraction was contaminated with 2-ketodeoxyoctonate and outer membrane proteins including the porin(s). The separation of these two membranes was not facilitated by variations in lysozyme treatment, cell age, presence or absence of flagella, or longer lipopolysaccharide chain length. Sodium lauroyl sarcosinate extraction resulted in an outer membrane fraction which contained some inner membrane contamination and produced multiple bands upon sucrose gradient centrifugation. Triton X-100 extraction removed the inner membrane from the outer membrane and Triton X-100/EDTA treatment extracted lipopolysaccharide-rich regions of the outer membrane which contained almost exclusively the *Campylobacter* porin(s). These data indicated that the inner and outer membranes of the *Campylobacter* cell envelope were very difficult to separate, possibly because of extensive fusions between these two membranes.

INTRODUCTION

Campylobacter spp. have become known worldwide as important human enteric pathogens (Walker *et al.*, 1986). With an increased ability to culture and identify these organisms, the study of these pathogens has naturally turned to understanding cell structure and virulence properties. Cell structures that have been recognized as virulence factors include the surface array of serum-resistant *C. fetus* (Blaser *et al.*, 1987) and the underlying lipopolysaccharide (LPS) layer, which has long polysaccharide chains (Perez *et al.*, 1985). Serum-sensitive strains of *C. fetus*, *C. coli* and *C. jejuni* all contain 'rough' LPS with short polysaccharide chains (Logan & Trust, 1984; Perez *et al.*, 1985). All of these campylobacters also lack Braun's lipoprotein, a structural feature of the *Escherichia coli* and *Salmonella typhimurium* cell wall involved in anchoring the outer membrane (OM) to the peptidoglycan layer (Logan & Trust, 1982). Other important surface antigens include the flagellum, its component protein (flagellin) having a 62 kDa molecular mass (Newell *et al.*, 1984; Logan & Trust, 1984), a 31 kDa acid-extractable protein in *C. jejuni* and *C. coli* (Logan & Trust, 1983) and the major OM protein (MOMP) at 44–47 kDa. True to the prediction of Logan & Trust (1984), the MOMP of *C. jejuni* is the porin protein, although the aqueous channel formed through the OM is very small in comparison to pores formed by other bacteria (Huyer *et al.*, 1986). Similarly, a mixture of the two MOMP's of *C. fetus* form a relatively large and a smaller pore, and it is presently thought that both MOMP's are porins (Huyer *et al.*, 1986).

Abbreviations: OM, outer membrane; MOMP, major outer membrane protein; IM, inner membrane; KDO, 2-ketodeoxyoctonate (3-deoxy-D-manno-2-ulosonate); SDH, succinate dehydrogenase.

The ability to isolate rapidly *Campylobacter* spp. OM is an essential step in the identification of species and for antigenic analysis. The sodium lauroyl sarcosinate (sarkosyl) extraction method of Filip *et al.* (1973) is used almost exclusively for this purpose in *Campylobacter* studies. The assumption is made that *Campylobacter* cells fractionate like *E. coli*, yielding pure OM with the inner membrane (IM) removed. This study examines the use of sucrose gradient fractionation to prepare OM of *Campylobacter* spp. and compares these preparations with detergent-extracted OM preparations. A comparison of these techniques provides some insight into the structure of the *Campylobacter* cell envelope.

METHODS

Bacterial strains and cultivation. Strains of *Campylobacter* used in this study included *C. jejuni* UA580 (NCTC 11168), the flagellate and aflagellate strains of *C. jejuni* 81116 (Newell *et al.*, 1984), a nalidixic acid resistant strain of *C. coli*, strain UA30 (Taylor *et al.*, 1983) and *C. fetus* strain UA60. *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* K12 were also used.

Campylobacter spp. were grown in Mueller Hinton medium (Difco) in a solid (1.8% agar, w/v) or liquid form. *P. aeruginosa* was grown in Trypticase Soy Broth (BBL) and *E. coli* K12 was grown in Luria Broth [1% (w/v) bactotryptone (Difco), 0.5% yeast extract (Difco), 0.5% NaCl], at 37 °C for 16 h. Liquid medium for *Campylobacter* culture (600 ml) was contained in 3 litre Fernbach flasks. The cultures were grown statically at 37 °C in an atmosphere enriched with 7–8% CO₂, at 85% humidity. *C. coli* and *C. jejuni* spiral-shaped cells were harvested from liquid medium after 24 h, whereas *C. fetus* spiral-shaped cells were harvested at 48 h. Predominantly coccoid cells were harvested from agar plates at 72 h. The cells were concentrated by centrifugation, washed once with 8 mM-Tris/HCl, pH 7.8, and resuspended in the same buffer.

Cell breakage. Cell-free extracts were prepared by disruption in a French pressure cell as described by Page & von Tigerstrom (1982).

Sucrose gradient fractionation. Cell envelopes were recovered from the cell-free extract on a cushion of 72% (w/v) sucrose according to Page & von Tigerstrom (1982). The cell envelopes (1.8 ml) were fractionated further on a step gradient (modified from Hancock & Nikaido, 1978) composed of sucrose (w/v): 72%, 1.2 ml; 60%, 2.0 ml; 50%, 2.0 ml; 45%, 2.0 ml; and 40%, 2.0 ml; in 8 mM-Tris/HCl, pH 7.8, and centrifuged (30000 r.p.m., 16 h, 2 °C) in a Beckman SW40Ti rotor.

Isolation of OM fragments. OM fragments also were isolated from cells grown on solid medium by the washing and sedimentation procedure of Logan & Trust (1982). This procedure involved the washing of cells (that were not deliberately broken) in cold Dulbecco phosphate-buffered saline, pH 7.4 (Oxoid) followed by low speed centrifugation to remove whole cells and a high speed centrifugation (100000 g, 2.5 h, 5 °C) to sediment OM fragments.

Detergent extraction. OMs were isolated by selective solubilization of IM in the cell-free extract with sarkosyl as described by Filip *et al.* (1973). Also OM were prepared by the Triton X-100 fractionation method of Schnaitman (1971) as described by Huyer *et al.* (1986).

Chemical determinations. Protein content was measured by the Lowry method or by the method of Bradford (1976). LPS was estimated as described by Osborn *et al.* (1972) with 2-ketodeoxyoctonate (KDO) as a standard. Succinate dehydrogenase (SDH) activity was measured as described by Osborn *et al.* (1972).

Electrophoresis. SDS-PAGE was conducted as described previously (Page & von Tigerstrom, 1982). The protein samples (20 µg protein per well) were heated in SDS and 2-mercaptoethanol for 5 min at 100 °C prior to electrophoresis (Page & von Tigerstrom, 1982). Proteins were stained with Coomassie brilliant blue R-250 as described by Fairbanks *et al.* (1971) and molecular masses were determined by comparison to authentic standards (Sigma): phosphorylase *a* (94 kDa), bovine serum albumin (68 kDa), γ-globulin H-chain (50 kDa), ovalbumin (43 kDa), γ-globulin L-chain (~24 kDa), and lysozyme (~14 kDa).

RESULTS AND DISCUSSION

Sucrose gradient fractionation

C. coli and *C. jejuni* (data not shown) membranes were resolved into four major fractions by sucrose gradient centrifugation. The profiles from these two species (24 h, spiral-shaped cells) were virtually identical in terms of A_{280} and relative density of each fraction. At the bottom of the gradient there was an opaque white band (A) which accounted for the majority of the A_{280} on the gradient (Fig. 1, Table 1). Proceeding up the gradient there was another opaque white band (B), an orange-white band (C), and finally a red-orange band (D).

Fig. 1. Separation of *C. coli* IM and OM fractions after sucrose gradient centrifugation. *C. coli* membranes (○) prepared after breakage in the French pressure cell (I), after sarkosyl extraction of cell envelopes (II) or released from washed whole cells (III) were separated on a sucrose step gradient (see Methods). Fraction (0.4 ml each) numbers 3–4 (A), 7–8 (B), 11–12 (C), and 16–18 (D) were collected from the bottom of the gradient and pooled for further analysis. *P. aeruginosa* ATCC 27853 (●) and *E. coli* K12 (□) cell envelopes also were fractionated on these gradients.

Table 1. Chemical analysis of sucrose gradient fractions of *C. coli* and *C. fetus* membranes

The data are representative of replicate sets of gradients. The results were reproducible between replicates by ± 5 –10%. Greater variation existed between duplicate trials but the pattern of fractionation and activities per fraction was constant for each species.

Organism	Fraction	Percentage of total A_{280}	Protein (mg ml ⁻¹)	SDH specific activity*	KDO [μ g (mg protein) ⁻¹]
<i>C. coli</i>	A	27.5	3.4	0.77	0.65
	B	11.3	5.6	1.36	1.58
	C	11.2	2.3	3.19	3.02
	D	11.6	2.0	10.08	1.78
<i>C. fetus</i>	A	22.5	2.4	9.8	2.63
	B	14.1	2.5	26.8	2.88
	C	12.4	1.7	57.9	2.82
	D	17.6	1.7	66.4	1.49

* Specific activity is expressed as $\Delta A_{600} \text{ min}^{-1} (\text{mg protein})^{-1}$.

The lower bands (A–C) contained KDO but this OM marker was not exclusively located in these fractions. Additionally all of the fractions contained some SDH activity, and indicated, in the case of *C. coli*, that IM cross-contaminated the OM fractions by approximately 5%, fraction A, 9%, fraction B, and 21% fraction C. This fractionation pattern was very similar to that obtained with *P. aeruginosa* PAO1 by Hancock & Nikaido (1978). When *P. aeruginosa*

Fig. 2. SDS-PAGE of *C. coli* (a) and *C. fetus* (b) membrane fractions from sucrose gradients. Lanes A–D correspond to the fractions in Fig. 1. Molecular masses of standard proteins are indicated on the left and right.

ATCC 27853 cell walls were fractionated on the sucrose gradients used in the present study, the same pattern of bands was obtained (Fig. 1). These bands, according to KDO and SDH activity (data not shown), corresponded to OM1 (peak fraction 3), OM2 (peak fraction 8), M (peak fractions 13–14) and IM (peak fractions 19–20), using the terminology and criteria of Hancock & Nikaido (1978). The three most dense bands: OM1, OM2, and M, were contaminated with IM material (as estimated by SDH activity) to the extent of 7%, 21%, and 31%, respectively, but the IM fraction contained essentially no KDO (data not shown; Hancock & Nikaido, 1978). *E. coli* K12 cell walls, on the other hand, were clearly separated on these gradients into OM (peak fraction 3) and IM (peak fractions 21–22; Fig. 1), and SDH activity indicated that the OM fraction was contaminated with IM material to the extent of $\leq 5\%$. From these results, it was tentatively concluded that the *Campylobacter* membrane fractions A, B, and C corresponded to OM material, cross-contaminated with IM material. It also was tentatively concluded that the uppermost fraction D, which contained the majority of the SDH activity, a red-orange (haem) colour, but some KDO, was the IM-enriched fraction.

Further resolution and separation of *Campylobacter* IM and OM fractions was not possible, despite the use of different sucrose concentrations in the gradient. The position of these bands was not affected by the presence or absence of lysozyme prior to sucrose gradient centrifugation or the use of sonic disruption rather than the French pressure cell (data not shown). Similarly, coccoid cells harvested at 72 h produced the same pattern of fractionation, with exact coincidence of fractions and identical A_{280} values as reported in Fig. 1 for the 24 h spiral-shaped cells. *C. fetus* A_{280} profiles from sucrose gradients always showed a more 'saddle-shaped' distribution with greater recovery of A_{280} in the IM fraction (D). The A_{280} profiles and protein contents per fraction also reflected the decreased content of fractions A and B in *C. fetus* (Table 1). The assays of SDH activity and KDO, however, indicated that the membrane fractions were cross-contaminated. Therefore the presence of 'smooth' LPS in *C. fetus* did not result in better resolution of IM and OM fractions.

Cross-contamination of the membrane fractions also was visible when the sucrose gradient fractions were analysed by SDS-PAGE (Fig. 2). The striking first impression of the gels was the

great number of protein bands seen in these fractions and the number of proteins common to both the IM and OM fractions. One of these proteins at approximately 70 kDa molecular mass, however, appeared to originate in the IM but was present in the OM as a contaminant (Fig. 2). The MOMP of *C. coli*, which is the porin (E. A. Worobec & G. Huyer, unpublished data), was concentrated in fractions B and C, but clearly was present in all the fractions. Flagellin, similarly, was concentrated in fractions B and C (Fig. 2). SDS-PAGE of the sucrose gradient fractions of *C. fetus* membranes also showed that the two MOMPs (presumed porins, Huyer *et al.*, 1986) at 45 kDa and 47 kDa were distributed through all the fractions. These proteins, however, were concentrated in fractions A and B. This 'movement' of the porins into the more dense fractions of *C. fetus* membranes was like that reported in the comparison of smooth- and rough-LPS mutants of *E. coli* and *S. typhimurium* (Ames *et al.*, 1974; Koplow & Goldfine, 1974). There also was a coincidence of the concentration of porin and LPS in the OM fractions of *C. coli* and *C. fetus* (Fig. 2 and Table 1). *C. jejuni* gave results very similar to *C. coli* (data not shown). Both flagellate and aflagellate strains of *C. jejuni* produced identical sucrose gradient fractionation and protein patterns upon SDS-PAGE, save the lack of flagellin (data not shown). Ames *et al.* (1974) suggested that membrane fractions with a density intermediate between OM and IM may exist because of flagellar attachment. Similar fractionation profiles between flagellate and aflagellate strains, however, indicated that this was not the reason for fractions B and C in *C. jejuni*.

From these data it was clear that the IM and OM of *C. coli*, *C. jejuni* and *C. fetus* were not being separated upon sucrose gradient centrifugation. This possibly was due to entrapment and cosedimentation of IM and OM vesicles, or due to physical adhesion of the two membranes (Osborn *et al.*, 1972; Schnaitman, 1970; White *et al.*, 1972). Two approaches were taken to attempt to resolve these possibilities: (a) the examination of OM fragments released during the growth of *Campylobacter* and (b) the selective removal of IM material by dissolution in the detergents sarkosyl or Triton X-100.

OM fragments released from whole cells

Sucrose gradient centrifugation of OM fragments released from whole cells of *C. coli* revealed the absence of fraction D and the presence of a band slightly less dense than fraction C (Fig. 1). The three bands, however, were contaminated with SDH activity and the protein profile on SDS-PAGE showed the presence of MOMP, flagellin, a great variety of minor proteins, and the 70 kDa IM protein (Fig. 3). Therefore, this material was cell envelope fragments rather than true OM fragments. This material possibly originated from the degenerate coccoid cells which are osmotically fragile (Moran & Upton, 1986) and frequently demonstrate plasmolysis and lysis (Moran & Upton, 1987; Ng *et al.*, 1985; Rollins & Colwell, 1986).

Treatment of cell envelopes with sarkosyl

OM prepared by sarkosyl extraction of *C. coli* cell envelope material also was composed of three types of membrane fragments that separated on sucrose gradients as fractions A, B and C (Fig. 1). The IM, fraction D was missing as expected. SDS-PAGE of these preparations showed the presence of many proteins, including MOMP and flagellin (Fig. 3). A small amount of SDH activity occurred in all the fractions (data not shown) but the 70 kDa IM protein was notably absent. The number of proteins present in fraction A (Fig. 2) was greatly reduced after sarkosyl extraction (Fig. 3) with a corresponding decrease in A_{280} (Fig. 1), which indicated that this fraction also had been significantly contaminated with IM material.

Triton/EDTA extraction

Triton/EDTA extraction of *C. coli* cell envelopes separated the MOMP from the other proteins present in the membrane mixture (Fig. 4). The Triton-soluble, presumptive IM fraction (Schnaitman, 1971), contained a variety of proteins, notably the presence of the 70 kDa protein and absence of flagellin and the MOMP. These latter proteins remained in the Triton-insoluble, presumptive OM material (Schnaitman, 1971). Further extraction with Triton/EDTA fractionated the Triton-insoluble material into the Triton/EDTA-insoluble fraction, which

Fig. 3

Fig. 3. SDS-PAGE of *C. coli* OM fragments and OM prepared by sarkosyl extraction. OM fragments washed from whole cells (1), OM prepared by sarkosyl extraction (2), and fractions from sucrose gradient fractionation of sarkosyl-extracted cell envelopes (3) were applied at 20 μ g protein per well, except in the case of lane 3D, which contained very little protein (see Fig. 1), where only <5 μ g protein could be applied to the gel. In (3), lanes A–D correspond to the fractions in Fig. 1. Molecular masses of standard proteins are indicated on the left.

Fig. 4

Fig. 4. Triton/EDTA fractionation of *C. coli* envelopes. Lanes: 1, Triton-insoluble OM material; 2, Triton-soluble IM material; 3, Triton/EDTA-insoluble OM material; and 4, Triton/EDTA-soluble OM material. The membrane material was applied at 20 μ g protein per well. Molecular masses of standard proteins are indicated on the left.

contained flagellin and other proteins, some of which were shared with the Triton-soluble fraction, and the Triton/EDTA-soluble fraction, which contained the MOMP (porin) as an essentially pure protein (Fig. 4). This Triton/EDTA fractionation procedure has been used previously to selectively extract the MOMPs (presumed porins) of *C. fetus* and the porin of *C. jejuni* (Huyer *et al.*, 1986).

Chemical analysis revealed that the Triton/EDTA-soluble fraction from *C. coli*, *C. jejuni* and *C. fetus* contained 7.8, 11.8, and 33.9 μ g KDO (mg protein)⁻¹, respectively. The Triton-soluble fraction contained 0 to 0.7 μ g KDO (mg protein)⁻¹ and the only detectable SDH activity. The Triton/EDTA-insoluble fraction contained a large amount of protein and relatively little KDO [1.3 to 3.4 μ g KDO (mg protein)⁻¹].

We conclude that the fractionation of *Campylobacter* membranes by sucrose gradient fractionation and sarkosyl extraction did not clearly eliminate IM contamination of the OM fractions. The appearance of multiple OM fractions after sucrose gradient fractionation was more like that obtained with *P. aeruginosa* (this study; Hancock & Nikaido, 1978) than with *E. coli* K12. In *Campylobacter* spp. and *P. aeruginosa* the multiple forms of OM appear to be due to IM contamination. However, in the case of *Campylobacter* the IM is significantly contaminated with OM porin protein(s) and KDO, while in *P. aeruginosa* the IM is essentially free of KDO and OM proteins (Hancock & Nikaido, 1978). This cross-contamination of membrane fractions was unlikely to be due to physical entrapment of membrane vesicles, because sarkosyl extraction removed the IM fraction, but did not result in a single OM fraction.

A possible explanation for these results is that the *Campylobacter* IM and OM are fused at numerous places, and that these in turn may stabilize the cell envelope of these lipoprotein-deficient cells. Sarkosyl extraction may remove IM 'patches' but may leave the membrane fusions in the OM preparation. This results in a complex OM preparation that contains many proteins, but only a few may be truly of OM origin. The other method of selective membrane solubilization, Triton X-100 extraction appeared to be more efficient in removing IM material containing SDH activity from the OM fraction. Triton/EDTA extraction then appeared to remove LPS-rich OM 'patches' containing porin, leaving the majority of the proteins including flagellin in the Triton/EDTA-insoluble material.

The coincidence of porin and LPS, and the distribution of porin through the sucrose gradient fractions is strongly suggestive of extensive adhesions between the OM and IM of the *Campylobacter* cell wall (Bayer, 1979; Smit & Nikaido, 1978). It is interesting that attempts to plasmolyse the spiral forms of *Campylobacter* spp. have been unsuccessful (L.-K. Ng, personal communication), which may be a consequence of the proposed extensive fusion between the IM and OM. Coccoid forms, however, frequently appear 'plasmolysed' in routine preparations (Buck, *et al.*, 1983; Ng *et al.*, 1985; Moran & Upton, 1987), but this 'plasmolysis' is accompanied by visible damage to both the OM and IM (Moran & Upton, 1987) and the leakage of cytoplasmic constituents (Moran & Upton, 1986). These observations also support the possibility of extensive fusions between the IM and OM and indicate that separation may be difficult or impossible without severe damage to membrane integrity. The distinction between viable but non-culturable coccoid cells (Rollins & Colwell, 1986) and non-viable coccoid cells may depend on the extent to which the OM and IM are torn apart during conversion to the coccoid form. The possibility of extensive adhesions between the IM and OM also may have important consequences on the uptake and export of hydrophobic compounds (as reviewed by Nikaido & Vaara, 1985) by these species of *Campylobacter*.

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