Antigenic shifts in serotype determinants of *Campylobacter coli* are accompanied by changes in the chromosomal DNA restriction endonuclease digestion pattern

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Summary. Changes in somatic (O) lipopolysaccharide (LPS) antigenic specificities of *Campylobacter coli* serostrains were observed after continuous laboratory subculture. Two serostrains (*C. coli* O34 and *C. coli* O48) lost O specificity and did not react with homologous or any of the available heterologous antisera. The *C. coli* serostrain for serogroup O5, after subculture, yielded a variant that had acquired a new specificity which was detectable with a heterologous antiserum. In a repeat experiment with the original isolate of the O5 strain, a second variant was obtained which had not only acquired the same new determinant but had, unlike the first variant, lost reactivity with the homologous antiserum. Immunoblot experiments with homologous and heterologous antisera indicated that changes in antigenic specificity were associated with the O side chains of the LPS molecules. Results of restriction endonuclease analysis of chromosomal DNA of the variants and their parents revealed minor differences in restriction patterns which suggested that *C. coli* is capable of undergoing genomic re-arrangements that lead to changes in LPS specificity and structure.

Introduction

Campylobacter jejuni and *C. coli* are among the leading causes of bacterial diarrhoea in man. A serotyping system based on thermostable antigens has been developed in our laboratory for these organisms^{1,2} and recent investigations have shown that the antigens are somatic (O) lipopolysaccharides (LPS).^{3–5} Forty-two serotypes of *C. jejuni* and 18 serotypes of *C. coli* have been identified by passive haemagglutination (PHA) tests. All *C. coli* serotype reference strains (serostrains) have been found to be of the smooth (S) type that have LPS with O side chains.⁶ In contrast, approximately one-third of *C. jejuni* serostrains that have been examined have S-type LPS and two-thirds have only low mol. wt LPS.⁵ The high mol. wt bands of *C. jejuni* and *C. coli* LPS chains are not observed in silver-stained gels (SDS-PAGE) but can be visualised in immunoblots, suggesting that their chemical structure is such that silver staining of the core material is inhibited in the presence of O side chains.⁵

We have observed that, during routine laboratory subculture, some strains lose their serotype determinants or gain new ones. This observation prompted the present study to investigate the stability of LPS antigens and the phenomenon of antigenic variation

that results in changes in the serospecificity of the campylobacter LPS antigens.

Materials and methods

Bacterial strains and culture conditions

The *Campylobacter* strains used were reference strains from the typing scheme described previously.^{1,2} They included *C. coli* strain PC330, the serostrain for serogroup O5; strain HSC-PC330, the same strain, but maintained by M. Karmali at the Hospital for Sick Children, Toronto; strain MK23, the original serostrain for *C. jejuni* serotype O5; and strain C88, an alternative serostrain for *C. jejuni* O5.

Bacteria were cultured routinely on blood agar (Columbia Agar Base, Oxoid, with oxalated horse blood 5%). Plates were incubated in CO₂ 7% v/v at 37°C for 48 h. Hippurate hydrolysis tests were done by the standard technique of Harvey and Greenwood.⁷ Thioglycollate Medium (Difco) was prepared according to the manufacturer's instructions.

Antisera

The two antisera used were serotyping antisera prepared in rabbits by intravenous injections with bacterial suspensions in saline of the serostrains MK23 and PC330 respectively.

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Passive haemagglutination assay

The PHA technique¹ was used to determine the titres of serotyping antisera against the serostrains and the variant strains. Briefly, antigenic extracts were prepared by harvesting the confluent bacterial growth from blood-agar plates into saline (NaCl 0.85% w/v), 1 ml/plate, and heating the suspension for 1 h at 100°C. After cooling to room temperature, the cell suspension was centrifuged (8000 *g* for 10 min) and the supernate containing the thermostable soluble antigen was removed. For titration of the antisera, the supernate was diluted 1 in 10 in isotonic phosphate-buffered saline (IPBS) (10 mM Na₂HPO₄, pH 7.0, in saline) and then mixed with an equal volume of washed sheep erythrocytes 1% v/v. After incubation at 37°C for 1 h, the sensitised erythrocytes were centrifuged (2000 *g* for 10 min), washed three times in the original volume of IPBS and resuspended in IPBS to 0.5% v/v. Two-fold dilutions of the antisera were made with a medimixer (Flow Laboratories, Inc., McLean, VA, USA) in microtitration plates (Dynatech Laboratories Inc., Alexandria, VA, USA) with U-shaped wells containing 25 µl of IPBS. Sensitised erythrocytes (25 µl) were added to each well, and the plates were incubated at 37°C for 1 h and then stored overnight at 4°C. Agglutination of the erythrocytes indicated a positive reaction and the highest dilution showing agglutination was taken as the titre. The initial dilution of the antisera was 1 in 40.

Isolation of antigenic variants

Serostrains were maintained in thioglycollate medium at 37°C for 1 month periods and then subcultured on to blood agar. After isolation of single colonies to ensure purity, cultures were checked for antigenic specificity, and then growth from a single colony was inoculated into fresh thioglycollate medium. Variants in LPS specificity were maintained in a solution of proteose peptone 10% w/v and glycerol 30% v/v at -70°C.

Proteinase K digestion of solubilised whole cells

The procedure of Hitchcock and Brown⁸ was modified as follows: growth from a single plate was removed with 5 ml of IPBS and diluted to an optical density of 0.3 at 600 nm. A volume of 1.5 ml was transferred to an eppendorf tube, the cells were centrifuged, and the pellet was resuspended in 200 µl of lysis buffer (glycerol 20% v/v, mercaptoethanol 5% v/v, sodium dodecyl sulphate (SDS) 4.6% w/v, bromophenol blue 0.004% w/v, 0.625 M Tris-HCl, pH 6.85). To ensure efficient lysis, the preparation was heated to 100°C for 10 min and, after cooling, 40 µl of lysis buffer containing proteinase K 2.5 mg/ml was added to each tube. Samples were incubated overnight at 37°C and then for 2 h at 65°C.

Electrophoresis and silver staining

Proteinase K-digested whole cells were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with the discontinuous buffer system of Laemmli⁹ and with a 5% stacking gel and a 10% separating gel. Electrophoresis was at a constant current of 35 mA until the tracking dye was within 1 cm of the bottom of the separating gel. The gels were then used for immunoblotting or were fixed and stained for LPS by the silver-staining method of Tsai and Frasch.¹⁰

Immunoblotting

After electrophoresis, gels were immersed in transfer buffer (25 mM Tris, 10 mM glycine, methanol 20% v/v) for 10 min at room temperature and then the LPS was transferred to pure nitrocellulose (pore size 0.45 µm, BioRad Laboratories, Richmond, CA, USA). Blotting was performed in the Trans-Blot system (BioRad) for 2.5 h at 65 V.¹¹ After transfer, the nitrocellulose sheets were soaked for 15 min in Tris-buffered saline (TBS; 20 mM Tris, 500 mM NaCl, pH 7.5) containing gelatin 3% w/v and then incubated overnight in TBS containing gelatin 1% w/v and rabbit antiserum diluted 1 in 50. The following day, the sheets were rinsed once in distilled deionised water (ddH₂O), washed twice in TBS with Tween-20 0.05% w/v for 10 min each, and then incubated for 3.5 h at room temperature in TBS with gelatin 1% w/v and biotin-conjugated goat anti-rabbit IgG (Cappel, Organon Teknica Corp., West Chester, PA, USA) 2 µg/ml. Blots were washed as described above, incubated in TBS with gelatin 1% w/v and avidin-peroxidase 1.7 µg/ml and washed once again. Colour was developed with the chloro-β-naphthol developing reagent from BioRad.

Preparation of chromosomal and plasmid DNA

The technique used to prepare total cellular DNA was described previously.¹² Briefly, confluent growth on 3–5 blood-agar plates was removed with 3 ml of IPBS and the cells were washed twice in the same buffer. After the final centrifugation, the cells were weighed (250 mg wet wt required) and resuspended in 4 ml of TE buffer (10 mM Tris-HCl, 100 mM sodium edetate, pH 8.5). Lysozyme (type II, Sigma) was added to a final concentration of 3 mg/ml and, after incubation at 37°C for 12 min, the cells were lysed by adding SDS to 1% final concentration. Contaminating RNA was removed by digestion with RNAase A (50 µg/ml) for 60 min at 37°C followed by an overnight protein digestion with proteinase K (0.5 mg/ml) and pronase (0.8 mg/ml). The DNA was extracted three times with an equal volume of phenol-chloroform, once with ether and then concentrated by ethanol precipitation. The final pellet was resuspended in 400–500 µl of sterile ddH₂O. The concentration of DNA and its purity were determined by optical density readings at

260 nm and 280 nm and the final concentration was adjusted to 1 µg/µl.

Restriction endonuclease digestion and agarose gel electrophoresis

The enzymes *Bgl*III, *Hind*III, *Bam*HI, *Pvu*II and *Hae*III were obtained from Boehringer-Mannheim and used in accordance with the manufacturer's instructions. The appropriate volume of DNA (containing 15–20 µg) was digested with the enzyme in buffer provided by the manufacturer in a total volume of 30 µl. Undigested samples were electrophoresed to determine the presence of plasmids. All preparations were electrophoresed in Tris-acetate edetate buffer overnight at 30V constant voltage. Gels were stained in ethidium bromide 1 µg/ml and destained in several changes of buffer. The mol. wt markers obtained from BRL (Gaithersburg, MD, USA) included a series of high mol. wt DNA fragments from 48.5 kb to 8.3 kb and a 1-kb DNA ladder from 12.2 kb to 298 bp.

Results

Isolation of antigenic variants

At the beginning of our studies on *Campylobacter*, the serostrains were maintained routinely in static culture in thioglycollate medium at 37°C for 1-month periods. From this state they could be retrieved more readily for day-to-day operations on blood agar than they could from the glycerol-salts solution kept at –70°C or from the lyophilised state. It was found, however, that the three reference strains of *C. jejuni* for groups O41, O45 and O50 could not be passaged at monthly intervals for more than 3–4 months in the thioglycollate medium without gradual loss of viability. Two strains (*C. coli* O34 and *C. coli* O48) that did remain viable either lost their O specificities or underwent a change in specificity that could not be detected by available typing antisera. However, the serostrain PC330 for *C. coli* group O5 was observed to undergo a change in specificity after a period of 7–10 months of monthly subculturing. The antigenically altered strain reacted strongly with antiserum against strain MK23, the serostrain for *C. jejuni* serogroup O5, whereas the parental strain reacted with the same antiserum only at the low titre of 1 in 40 (table I, part A). The O5 serostrains of these two species cross-react but are not identical. Therefore, the interpretation given to this observation was that during subculture strain PC330 had undergone antigenic variation by acquiring a determinant (epitope) possessed by the *C. jejuni* group O5 antigenic complex that was not present in the *C. coli* O5 complex.

To study this antigenic variation further and to assess the reproducibility of the phenomenon, another trial of subculturing in thioglycollate medium was done with *C. coli* strain HSC-PC330. This was the

Table I. Serotyping of *C. coli* strains and their variants

Strain	PHA titres with antisera against strains	
	PC330	MK23
<i>A. Original passage</i>		
PC330	40 960	40
PC330V*	40 960	2560
<i>B. Repeat trial</i>		
HSC-PC330	40 960	40
HSC-PC330V*	1280	2560

*Variants isolated after 7–10 months of subculture.

original isolate, maintained in another laboratory, from which our strain PC330 was obtained. After subculturing for 10 months, a variant (HSC-PC330V) was isolated that differed from the parental strain in both a loss of reactivity with PC330 antiserum and a gain of reactivity with the MK23 antiserum (table I, part B). The titres recorded for the two antisera over the 10-month period are shown in table II.

The differences between the parental strains and their variants and the differences between the two variants were not due to mixed colony types as cultures of each, which had been derived from single colonies, gave virtually identical results in serotyping to those shown in table I. The two variants were shown to have a cell morphology typical of *Campylobacter* and negative hippurate hydrolysis reactions characteristic of *C. coli*.

Table II. Serotyping of *C. coli* HSC-PC330 subcultured in thioglycollate medium over a 10-month period

Number of months of subculture	PHA titres with antisera against strains	
	PC330	MK23
0	40 960	40
4	40 960	320
5	40 960	320
7	40 960	320
10*	1280	2560

*Variant isolated after 10 months of subculturing was designated HSC-PC330V.

Characteristics of LPS

Washed whole-cell suspensions of the original *C. coli* strains PC330 and HSC-PC330, the two variants PC330V and HSC-PC330V, and the *C. jejuni* serostrains MK23 and C88, were solubilised and then treated with proteinase K. The LPS was electrophoresed and silver-stained (fig. 1). The results showed the absence of high mol. wt bands in all strains,

Fig. 1. Silver-stained SDS-PAGE gel of proteinase K-digested whole-cell lysates. Lane 1, *C. coli* PC330; 2, *C. coli* PC330V; 3, *C. coli* HSC-PC330; 4, *C. coli* HSC-PC330V; 5, *C. jejuni* MK23; 6, *C. jejuni* C88.

consistent with earlier reports that *C. jejuni* and *C. coli* high mol. wt LPS is not stained by this method.^{5,6} The parental *C. coli* strains and their variants had two low mol. wt bands of virtually the same mol. wt (fig. 1, lanes 1–4). The changes in serotyping results observed for the two variants were not reflected in any obvious changes in the mobility of core LPS. The *C. jejuni* strains MK23 and C88 (fig. 1, lanes 5 and 6) had low mol. wt LPS bands quite distinct in migration rates from those of the *C. coli* strains.

Similar gels were transferred to nitrocellulose and immunoblotted with antiserum to *C. coli* strain PC330. The LPS from each strain of *C. coli* and *C. jejuni*

reacted with this antiserum (fig. 2B). Because the PHA titres are based primarily on the presence of IgM whereas the reactions observed in immunoblotting are exclusively due to IgG, and because the relative sensitivities of each test are different, absolute comparison of results are inappropriate. However, there was complete consistency in that high and low titres in PHA always corresponded to positive and negative reactions by immunoblotting. The low mol. wt core bands visualised in silver-stained gels of strains PC330, HSC-PC330 and the two variants reacted very strongly with the antiserum to strain PC330 (fig. 2B). Furthermore, a series of 10–12 high mol. wt bands were observed in the parental *C. coli* strains reflecting the presence of O-polysaccharide side chains of varying length (fig. 2B, lanes 1 and 3). The variants that showed a decrease in PHA titres with this antiserum (table II), still displayed this high mol. wt material, but the bands were much less intense than were the bands from the other strains, consistent with a loss of a reactive determinant (fig. 2B, lanes 2 and 4). No obvious change in mol. wts of these O-antigen units was noted when the original and the variant strains were compared. For *C. jejuni* strain MK23, the presence of banding reflecting smooth type LPS was observed but with fewer repeat units and with a distribution distinct from the LPS profiles of the *C. coli* strains (fig. 2B, lane 5).

Immunoblots with antisera against *C. jejuni* strain MK23 (fig. 2, panel A) reacted strongly with homologous LPS in both core and O-side chain regions, as expected from the PHA results (table I). Moreover, it also reacted with the S-LPS bands found in *C. coli* PC330V (weakly) and *C. coli* HSC-PC330V (fig. 2A, lanes 2 and 4), but not with LPS from the parental strains (fig. 2A, lanes 1 and 3). These results correlated with the PHA results (table I) which indicated the presence of a new determinant in the variant strains.

Fig. 2. Immunoblots with antisera to *C. jejuni* MK23 (Part A) and *C. coli* PC330 (Part B) and proteinase K-digested whole-cell lysates. Lane 1, *C. coli* PC330; 2, *C. coli* PC330V; 3, *C. coli* HSC-PC330; 4, *C. coli* HSC-PC330V; 5, *C. jejuni* MK23.

Restriction endonuclease mapping of chromosomal DNA

Total DNA was prepared from all of the *C. jejuni* and *C. coli* strains. Uncut preparations of DNA were electrophoresed to determine the presence of plasmids but none was detected in any of the strains. The chromosomal DNA was digested with five different restriction endonucleases and the restriction patterns of the original isolates and their variants were compared after the DNA fragments were electrophoresed in agarose. The enzymes *Pvu*II and *Bgl*II produced identical restriction patterns for all of the *C. coli* strains (results not shown). *C. jejuni* strain MK23 had a pattern different from the *C. coli* strains. The use of enzyme *Bam*HI revealed a difference in fragments for *C. coli* PC330 and the antigenic variant PC330V (results not shown). A band of c. 8.5 kb was present in the variant but absent in the pattern of the parent, and this clearly distinguished the two strains.

The use of the enzyme *Hind*III revealed a difference in the chromosomal DNA restriction patterns between strain HSC-PC330 and its variant HSC-PC330V (results not shown). The latter did not have a 5.2-kb fragment present in the former. The restriction patterns for the other *C. coli* strains (PC330 and PC330V) were identical to the pattern shown by DNA from strain HSC-PC330 (results not shown). *Bam*HI and *Hind*III produced restriction patterns for *C. jejuni* strain MK23 that were very different from the patterns shown by the *C. coli* isolates (results not shown).

Of the five restriction endonucleases used in this study, *Hae*III yielded the most clearly resolved chromosomal DNA restriction patterns (fig. 3). The *C. jejuni* strains were clearly shown to be different (fig. 3, lanes 5 and 6) from the *C. coli* strains (fig. 3, lanes 1–4). With this enzyme, the parental strains, *C. coli* PC330 and *C. coli* HSC-PC330, also yielded patterns that were indistinguishable and were thus confirmed to be the same strain (fig. 3, lanes 1 and 3). However, of greater importance was the finding that the patterns of their progeny, *C. coli* strains PC330V and HSC-PC330V were each different from the patterns of their parents and different from each other in the high mol. wt region (15–32 kb), as indicated by the arrows in fig. 3. This was taken as evidence that variants with different LPS specificities possessed differences in

their genomic structures that could be detected by restriction endonuclease analysis with the appropriate enzyme.

Discussion

Serotyping systems are important tools in the field of clinical microbiology and epidemiology. They should be simple to apply, able to differentiate a variety of isolates, and be based on stable antigens. The antigenic structure of the O antigen of many gram-negative organisms has been reflected in serological diversity, as most of the O antigens have groups which are immunodominant and some have more than one group antigen.¹³ These antigens are lipopolysaccharides (LPS) and are present in large quantities, accessible on the surface of live or dead whole cells, easily extracted and highly immunogenic.¹³ Although O antigens are generally recognised as the most stable of the antigens that can be used for serotyping, they are also known to undergo occasional changes in specificity when subjected to certain biological influences or agents.^{14–16} A loss or gain of a determinant in the O-side chain repeat unit can be mediated by a lysogenic bacteriophage or by regulation of gene expression^{13,14,16} and usually involves either methylation or glycosylation.^{14,16} The LPS molecule can also vary from the S-type to the R-type, thereby becoming untypable with the antiserum prepared against the parental S-type.^{17,18} Variants of this kind may have been the basis for the loss of specificity of *C. coli* sero strains O34 and O48. Alternatively, they may have acquired new specificities that would require the preparation of antisera against the variants to demonstrate the new specificities. This was not done in the present study, but the two variants that were produced from subcultures of the *C. coli* O5 strain (PC330 and HSC-PC330) were characterised by the PHA technique, SDS-PAGE coupled with immunoblotting, and by restriction endonuclease mapping of chromosomal DNA.

For all strains of *C. coli* examined, two types of LPS molecules have been demonstrated.⁶ The first is of low mol. wt and is visualised after SDS-PAGE by the silver-staining procedure of Tsai and Frasch.¹⁰ Although the chemical structure of these molecules has not been determined, it is thought that they consist of lipid A linked to core oligosaccharides. LPS of approximately the same mol. wt from enterobacteria is of the R-type, without O-side chains, or the SR-type, with a single repeat unit of the O-side chain.^{5,6,19} The other type of LPS molecules from *C. coli* and *C. jejuni* are of high mol. wt and show the characteristic ladder-like pattern of electrophoresed S-LPS of the enterobacteria.^{8,20,21} However, a significant difference between the S-LPS from enterobacteria and *Campylobacter* is that the former can be visualised by silver staining^{8,10} but the latter can be seen only in immunoblots.⁵ An explanation for the inability to

Fig. 3. Agarose gel electrophoresis of restriction endonuclease digests (*Hae*III) of chromosomal DNA from strains of *Campylobacter*. Lane 1, *C. coli* HSC-PC330; 2, *C. coli* HSC-PC330V; 3, *C. coli* PC330; 4, *C. coli* PC330V; 5, *C. jejuni* MK23; 6, *C. jejuni* C88; 7, high-mol. wt markers; 8, low-mol. wt markers.

detect these molecules with silver stain has not been forthcoming and awaits a detailed chemical analysis. Although some strains of *C. jejuni* bear only the low-mol. wt type LPS, they still exhibit the necessary antigenic specificities that enable them to be serotyped.^{5,6,19}

It is interesting that the two variants isolated in this study each possessed both low- and high-mol. wt LPS and that changes from the parental LPS consisted of readily detectable changes in high-, but not in low-mol. wt LPS. Therefore, the changes in antigenic specificity could arise through alterations in the O-side chains. That such alterations did occur was demonstrated by the changes in antigenic specificity detectable by PHA and immunoblotting.

There were no changes in plasmids. None of the strains maintained any extra-chromosomal DNA, including phage DNA, suggesting that the alterations observed were not plasmid-mediated.

To gain insight into the basis of the phenomenon of antigenic variation, restriction endonuclease mapping of chromosomal DNA was performed on parent and variant strains. It was clear that, although the two parents were identical, each of the variants showed a novel restriction pattern. However, these differences were limited to a small number of restriction fragments. Although this does not prove conclusively that the development of the variants involved genetic changes, it certainly indicated that such a possibility exists. Two observations pointed to a two-step process. The variant strain PC330V gained a new specificity without an accompanying loss of an original specificity, whereas the variant strain HSC-PC330V not only acquired a new specificity, but also lost an original specificity. This supports the conclusion that gain and loss of the specificities is mediated by separate events. Moreover, the observation that the restriction maps of the two variants differ in several fragments (see fig. 3) suggested that the genomic alterations that led to the two variants were not the same.

To our knowledge this is the first report of variations in *C. coli* leading to changes in LPS specificity and structure. Other researchers found that *C. coli* strains had undergone chromosomal re-arrangements when antigenic shifts occurred in the flagellar protein.²² The variants described in the present study will serve as the source materials for more extensive studies to determine the genetic basis for changes in LPS. However, it should be noted that the variants were derived through laboratory manipulations and do not indicate a frequency of occurrence that significantly affects the reliability of the serotyping system for reference work, particularly when the serostrains are maintained in the lyophilised state.

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