FLAGELLA PHASE VARIATION IN THE THERMOPHILIC CAMPYLOBACTERS

by

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ABSTRACT

The purpose of this study was to examine the contribution of flagella to the widely used Lior heat-labile serotyping scheme for thermophilic Campylobacters. The serotype chosen for examination, serotype 8, contains strains of both _Campylobacter jejuni_ and _Campylobacter coli_ and is commonly isolated from man. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of cultures of strains belonging to the Lio 8 serogroup, showed that many of the strains produced flagellin molecules of two different molecular weights (MW). Radio-immunoprecipitation experiments with the Lio 8 typing antiserum revealed that while most Lio 8 strains produced flagellin which reacted with Lio 8 antiserum, some strains produced flagellin which did not react with this antiserum. This indicated that while the Lio 8 antiserum contained a high titer of serospecific antibodies to flagellin the serotype determinants for the Lio 8 serogroup were not carried on the flagella.

Immune-electron microscopy with Lio 8 antiserum showed that some cultures were producing two antigenically distinct flagella. This observation together with the ability of strains to produce flagellins of different MW suggested that in _C. jejuni_ and _C. coli_ flagella were subject to antigenic phase variation. To show that a strain could switch from the production of one flagellin antigenic type to the production of a different flagellin antigenic type, cells originating from a single clone were selected which exhibited motility in the presence of Lio 8 antiserum. By SDS-PAGE analysis, flagellin produced

before the switch (phase 1) was found to have a MW of 61,000, while flagellin produced after the switch (phase 2) had a MW of 57,000. This flagellin switching was shown by three different strains. In the case of the Lio 8 serogroup type strain VC 167, the rate of the phase 1 to phase 2 (1.9×10^{-5}) switch was over ten times higher than the rate of the phase 2 to phase 1 switch (1.1×10^{-6}). Although the two flagellins reacted with polyclonal antiserum to non surface-exposed conserved Campylobacter flagellin epitopes and to a monoclonal antibody which recognized a cross reactive internal Campylobacter flagellin epitope, only phase 1 flagellin reacted with Lio 8 antiserum by radioimmune-precipitation, enzyme-linked immunosorbent assay, or by immune-electron microscopy. Absorption experiments confirmed that the serospecific epitopes were exposed on the surface of the native phase 1 flagella filament. Biochemical analysis of the two flagellin molecules revealed significant structural conservation. The N-terminal amino acid sequences were identical for the first 22 residues, and peptide mapping showed many conserved peptides. However, the flagellins were different in MW and amino acid composition, and also contained unique pepides.

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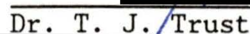

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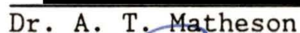

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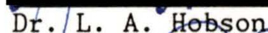

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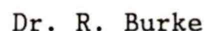

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LIST OF ABBREVIATIONS

Amino acids

Ala (A)	Alanine
Arg (R)	Arganine
Asn (R)	Asparagine
Asp (N)	Aspartic acid
Asx (B)	Asparic acid or asparagine
Cys (C)	Cysteine
Gln (Q)	Glutamine
Glu (E)	Glutamic acid
Glx (Z)	Glutamine or glutamic acid
Gly (G)	Glycine
His (H)	Histidine
Ile (I)	Isoleucine
Leu (L)	Leucine
Lys (K)	Lysine
Phe (F)	Phenylalanine
Pro (P)	Proline
Ser (S)	Serine
Thr (T)	Threonine
Trp (W)	Tryptophan
Tyr (Y)	Tyrosine
Val (V)	Valine
Met (M)	Methionine
AMP	Adenosine monophosphate

LIST OF ABBREVIATIONS CONTINUED

BHI	Brain heart infusion broth
CBA	Chocolate blood agar
CJT	*Campylobacter jejuni* toxin
CHO	Chinese hamster ovary cells
CT	Cholera toxin
DNase	Deoxyribonuclease
ELISA	Enzyme linked immunosorbent assay
GE	Glycine extract
HPLC	High performance liquid chromatography
IFAT	Immunofluorescence of living cells
Kdal	Kdaltons
Lio 8	Lior 8
LT	*E. coli* heat-labile toxin
LPS	Lipopolysaccharide
MW	MW
NARTC	Nalidixic acid resistant thermophilic Campylobacters
nt	Not tested
OM	Outer membrane
PHB	Polyhydroxybutyric acid
% G + C	Percent guanine plus cytosine
PMSF	Phenylmethylsulfonyl floride
RIP	Radio-immunoprecipitation
SDS-PAGE	Sodium dodecyl sulfate

LIST OF ABBREVIATIONS CONTINUED

	polyacrylamide gel electrophoresis
TCA	Tricarboxylic acid
TFA	Trifluoroacetic acid
TSB	Trypticase soy broth
v/v	Volume per volume
wc	Whole cells
wt/v	Weight per volume

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INTRODUCTION

The genus Campylobacter is composed of small gram negative, microaerophilic bacteria which are curved or spiral rods. These organisms can be found in the mammalian intestinal tract, oral cavity and reproductive tract and are responsible for a variety of diseases including abortion in cattle and sheep, and dysentary in swine (100). In man the thermophilic species Campylobacter jejuni and Campylobacter coli are a major cause of acute enteritis and are considered an important human pathogen (100). Indeed recent studies have revealed that gastrointestinal illness is more often caused by Campylobacter than the more widely known enteric pathogens, Salmonella and Shigella (12,22,23).

Because of the widespread occurence of Campylobacteriosis, serotyping schemes have been developed to facilitate epidemiologic studies. The serotyping schemes are based on both heat-stable and heat-labile surface antigens (10,63,84). Penner and Hennessy (84), for example, have serotyped C. jejuni and C. coli on the basis of heat-extracted soluble thermostable antigens. These antigens are detected using the passive hemagglutination technique. Lior et al. (63), on the other hand, have developed a serotyping scheme for Campylobacter based on slide agglutinations of live bacteria with absorbed antisera. The Lior scheme allows for the detection of heat-labile antigenic factors, but the molecular basis for the serospecificity of the scheme is not known at this time.

Campylobacter cells have a single polar flagellum situated at one end of the cell and move in a characteristic darting, corkscrew-like

manner (100). Usually these monotrichous flagella have no flagellar sheath and thus their flagellin protein is exposed (100) and likely to be immunogenic. Studies on Campylobacter flagella have been limited. Prior to this thesis study, the best described flagellum of the genus Campylobacter was that of Campylobacter fetus. These earlier studies were focused primarily on the antigenicity of the C. fetus flagellum and although the C. fetus flagellin had been partially purified, little detailed biochemistry was performed. In the case of C. jejuni and C. coli, studies by Logan and Trust (65) have shown that the flagellum protein was a major surface antigen, and as such may play a role in the Lior serotyping scheme.

This study was directed at one of the most common Lior serotypes, Lio 8. This serotype contains strains of both C. jejuni and C. coli. The objective of the study was to examine the role of flagella in the Lio 8 serotype. Indeed the study showed that while flagella of some strains belonging to serogroup Lio 8 reacted with the Lio 8 serotyping antiserum, the epitopes which serve as the serodeterminants for this serogroup were not flagella borne. Importantly, during the course of the investigation, the ability of certain strains to produce two antigenically distinct species of flagellin was observed. This is the first report of antigenic phase variation in the Genus Campylobacter, and a description of the phase variation together with a biochemical and immunochemical characterization of the antigenically distinct flagellin molecules is contained in this thesis.

LITERATURE REVIEW

The Genus Campylobacter

Taxonomy. *Campylobacter* and *Spirillum* comprise the family *Spirillaceae* (54). These two genera can be distinguished on the basis of the number of polar flagella, the ability to accumulate polyhydroxybutyric acid (PHB), and the % G + C composition of their DNA (46). *Campylobacter* have a single polar flagellum, cannot accumulate PHB and have a low % G + C content of 29-38% (54). *Spirillum*, however, have many flagella, accumulate PHB and have a % G + C content of 38-65% (54). Members of the Genus *Campylobacter* are curved, S-shaped rods 1.5-3.5 um long by 0.2-0.4 um wide (100). They are gram-negative, non-sporulating, microaerophiles which require an atmosphere of 10% CO_2 and 5% O_2. The ends of the cells are pointed. Cells from old cultures often become coccoid and are non-viable in this form. Campylobacters have a single polar flagellum at one or both ends of the cell. The flagellum is 2 to 3 times the length of the cell, is not sheathed and causes the cell to move in a corkscrew-like motion. *Campylobacter* do not ferment or oxidize carbohydrates and require complex media containing both amino acids and TCA cycle intermediates for growth (100). Species of *Campylobacter* are first divided on the basis of catalase activity (14) with *C. fetus*, *C. coli*, *C. jejuni*, *Campylobacter laridis* (NARTC) and *Campylobacter fecalis* being catalase positive (46). The catalase negative species include *Campylobacter sputorum*, and *Campylobacter concisus* (46).

Campylobacter species have been known since 1909 when *Vibrio* (now *Campylobacter*) *fetus* was isolated from aborted cattle and sheep (14).

Shortly thereafter, *C. fetus* was isolated from humans, as well as other animals (100). In 1957, King identified two serologically and biochemically different groups of *V. fetus* (48). She called the bacteria which grew best at 42°C "related vibrios" and the others *V. fetus*. The taxonomy of these thermophilic Campylobacters has been based on a few phenotypic characteristics and remains controversial.

As *V. fetus* and the "related vibrios" did not ferment glucose and differed in DNA composition from vibrios, Veron and Chatelain proposed the genus *Campylobacter* (111). The "related vibrios" became *C. jejuni* and *C. coli* and *V. fetus* became *C. fetus*. *C. fetus* is characterized by the following: it gives positive catalase, oxidase, and nitrate tests; no fermentation or oxidation of glucose, no H_2S production in triple sugar iron agar, growth at 25°C and no growth on agar containing 1% glycine (54). *C. coli* and *C. jejuni* give positive catalase, oxidase, and nitrate reactions, no fermentation or oxidation of glucose, and no H_2S production (54). They differ from *C. fetus* in that they do not grow at 25°C, they grow in 1% glycine, they have lactobacillic acid in their membrane, they are sensitive to nalidixic acid and resistant to cephalothin (35,54).

Pathogenicity. *C. fetus* is the cause of an infectious disease of cattle resulting in abortion and reproductive problems characterized by inability of heifers and cows to become pregnant (14). Transmission is venereal with *C. fetus* being isolated in the reproductive organs. *C. fetus* also causes abortion in sheep (14). The disease is generally acute with abortion occurring in the later stages of pregnancy. *C.*

fetus is also an infrequent opportunistic pathogen in man, causing septicemia and other illnesses (68).

C. coli has been found to be an important pathogen in sheep causing spontaneous abortions (100). *C. coli* and *C. jejuni* have been found to cause dysentery in swine and are common agents of acute enteritis in man (68). These species, especially *C. jejuni*, are important causes of diarrheal illnesses on all continents. In developing countries, *C. jejuni* has been isolated from the stools of 3 to 14% of patients with diarrhea, and was rarely isolated from healthy persons (6,16,97). In South Africa and Bangladesh, 40% of children 9 to 24 months of age excreted *C. jejuni* (7,11). In the United Kingdom, 9500 *C. jejuni* diarrhea isolates were reported in 1981, exceeding isolates of *Shigella* and approaching that of *Salmonella* (21). Other studies have shown that *C. jejuni* isolates can even exceed *Salmonella* isolates (12,22). Outbreaks of *C. jejuni* are also common in British Columbia (76).

Transmission of *C. jejuni* occurs by the fecal-oral route through contaminated food and water or by direct contact with fecal material from infected animals or persons. Most illnesses occur from one to seven days after exposure to the organism, with the incubation period depending upon the size of the inoculum (9). The sites of infection include the jejuneum, ilium, and colon. The infection symptoms include diarrhea, abdominal pain, malaise, fever, nausea, and vomiting (97). Usually the infection is self-limiting, however when necessary, erythromycin is the antibiotic of choice. *Campylobacter*-like organisms have also been isolated from gastric biopsy specimens, and peptic ulcers.

Toxins. C. jejuni appears to produce two toxins, an enterotoxin and a cytotoxin. The enterotoxin (CJT), is thought to be heat-labile, to raise intracellular cyclic AMP levels, to cause cytotonic changes in Chinese Hamster Ovary cells (CHO cells), and to induce fluid secretion in ligated rat ileal loops (49,50,51). This enterotoxin is also believed to be functionally and immunologically related to both cholera toxin (CT) and E. coli heat-labile toxin (LT)(49).

Enzyme-linked immunosorbent assays (ELISA) indicate that this toxin is immunologically similar to LT and CT (49). Some evidence also suggests that CJT contains a subunit related to the B subunit of CT and LT (49). CJT reacts in ELISA's with the GM1 ganglioside (49), and by incubating CJT with GM1 the toxin's cytotoxic response in tissue culture assays is inhibited (49). The toxin can be purified by affinity to the GM1 ganglioside (52), and immunization of rats with LT B subunit protects mice against crude CJT in ligated ileal loops (49). However, DNA colony hybridization studies have shown that the gene for CJT is not closely related to genes coding for CT or LT, suggesting that while the toxins are structurally different, they show similarities in their 3 dimensional shape.

Some C. jejuni strains also produce a cytotoxic response in various culture systems (51,117) and this toxin is also believed to be heat-labile. The pathogenic significance of this cytotoxin has not been determined, but may be involved in the dysentary-like symptoms observed in some infections. Other investigators (69,112) have concluded that no cytotoxin exists as none has been demonstrated in cell lysates.

Typing of the Genus Campylobacter

Biotyping. The Campylobacters are difficult to separate biochemically. However, those characteristics which are different have been utilized in a number of biotyping schemes. The first and still widely used biotyping scheme proposed was that of Skirrow and Benjamin (98,99). This was based on nalidixic acid sensitivity, hippurate hydrolysis, and H_2S production in an iron-containing medium. C. fetus gave negative results for both hippurate hydrolysis and H_2S production, while all C. laridis strains were resistant to nalidixic acid, negative for hippurate, and positive for H_2S. All hippurate positive strains were regarded as C. jejuni, while nalidixic acid sensitive and hippurate negative strains were regarded as C. coli. C. jejuni strains were further divided into two biotypes according to their ability to produce H_2S. C. jejuni biotype 1 (H_2S positive) is the type most often isolated from patients with acute enteritis but C. jejuni biotype 2 (H_2S negative) and C. coli have also been found. C. laridis strains have also been isolated in man.

A second commonly used biotyping scheme for thermophilic Campylobacters is by Lior (61). This scheme is based on a rapid hippurate hydrolysis test (40), rapid H_2S production in iron-bisulfate-pyruvate medium and DNA hydrolysis in modified DNase test agar. The Lior biotyping scheme separates three species of thermophilic Campylobacters and subdivides them into four biotypes of C. jejuni, two biotypes of C. coli, and two biotypes of C. laridis. Additional biotyping schemes have been suggested by other investigators.

For example, Hebert *et al*. (35) developed a scheme for *C. jejuni* based on hippurate hydrolysis, DNA hydrolysis, and growth on charcoal-yeast agar. In using these criteria 8 biotypes of *C. jejuni* were defined and differentiated from other *Campylobacter* strains. Unfortunately this scheme was only useful for *C. jejuni* and does not separate *C. coli* from *C. jejuni*. Roop *et al*. (87) developed a scheme capable of distinguishing between *C. jejuni* and *C. coli* strains. Alkaline phosphatase and deoxyribonuclease (DNase) activity and hippurate hydrolysis were used to assign *C. jejuni* strains to four biovars while alkaline phosphatase, DNase activity, and the ability to grow on minimal media, were used to place *C. coli* into four biovars.

Wong (115) grouped Campylobacters based on their reaction patterns with lectins. These reactions were found to be strain specific, reproducible, and unaffected by heat-stable antigens. Other schemes have included the examination of soluble protein extracts by PAGE (30), and auxotyping in which a chemically defined medium developed for *Neisseria gonorrhoeae* was modified to support the growth of *Campylobacter* strains (108). These various schemes have not received wide acceptance.

DNA studies have also been undertaken to show that *C. jejuni*, *C. coli* and *C. fetus* are separate species. For example, Belland and Trust (4) examined DNA base compositions, DNA:DNA hybridizations of S1 endonuclease digestions, and thermal stability of homologous and heterologous double stranded DNA to show that the strains studied comprised three species: *C. coli*, *C. jejuni* and *C. fetus*. Hebert (34),

and Harvey and Greenwood (33) also used DNA-DNA hybridizations to show that the *Campylobacter* species designations were genetically accurate. Analysis of restriction endonuclease digests of *Campylobacter* DNA has also been used to distinguish among species (20). Hybridization experiments with a DNA probe were also used by Steele (102) to determine species relatedness.

Serotyping. The problem with all of the biotyping schemes is that they are not sufficiently discriminating for epidemiological purposes. For example they cannot be used to accurately trace an outbreak. Therefore, serotyping systems have become widely used. Two different approaches have been taken with respect to serotyping. One is based on heat-stable antigens the other is based on heat-labile antigens.

The first *Campylobacter* to be studied serologically was *C. fetus*. Berg *et al*. (5) described three serotypes using both slide and tube agglutinations. The antigens involved in the reaction were thought to be somatic (O) antigens and called A, B, and C. Subclassification was then accomplished by preparing antisera against several thermostable antigens. It was then discovered that soluble heat-stable *Campylobacter* antigens could be heat-extracted from *C. fetus* and absorbed to mammalian erythrocytes (10). This allowed for the development of a passive hemagglutination test, and Bokkenheuser (11) showed that this test was more sensitive than bacterial agglutination. Subsequent studies by McCoy showed that the O antigen could be extracted with trichloracetic acid (70).

Today most attention with serotyping has focused on the thermo-

philic Campylobacters. The most widely used methods are those developed by Penner and by Lior. The serotyping scheme first reported by Penner and Hennessy (84) employs passive hemagglutination based on soluble heat-stable antigens. In this method, antigenic material was extracted from C. jejuni strains by heating bacterial suspensions in saline at 100°C, and by exposure to ethylendiaminetetra-acetic acid. The antigens extracted were stable at 100°C, produced specific antibodies in rabbits and were used to sensitize sheep erythrocytes for agglutination reactions with antibodies. Over 50 serotypes are found in the Penner scheme, and findings by Mills et al. (77) indicate that the scheme appears to be based on lipopolysaccharide (LPS) composition. In other Gram-negatives, heat-stable serotyping schemes are also based on LPS. LPS are heat-stable molecules located in the outer leaflet of the outer membrane (OM). In the case of Enterobacteriaceae they contain a distal O polysaccharide chain, and a core-polysaccharide region linked to lipid A which is embedded in the membrane (67). The O polysaccharide chain contains repeating units of 2-4 saccharides and is strongly antigenic.

In contrast to the Enterobacteriaceae, Logan and Trust (64) found that the LPS of C. jejuni and C. coli is of low MW, suggesting that heat-stable serotypic differences due to LPS are based on different carbohydrate compositions of core LPS. This is a novel finding that so many serotypes are based on low MW LPS. In contrast LPS of C. fetus exhibited O antigen polysaccharide chains of intermediate chain length. Other investigators (77,80,85,86) have confirmed that C. coli and C.

jejuni LPS is devoid of long O polysaccharide side chains. This low MW LPS was shown to confer heat stable serospecificity as antibodies to LPS of one strain did not react with heterologous LPS (66). The molecular basis for the differences seen in antigenicity of LPS between strains is unclear at this time, but may be due to changes in lipid structure, or the extent of phosphorylation, or to variations in polysaccharide structure. Mills et al. (53) extracted and purified LPS from strains of C. jejuni, and showed that the LPS could sensitize sheep erythrocytes and could be successfully employed in the passive hemagglutination Penner serotyping assay.

Another widely used serotyping scheme was developed by Lior et al. (62,63). This is used to serotype C. coli and C. jejuni and is based on heat-labile antigenic factors. In this method antisera produced to formalinized whole cells are absorbed with homologous heated and heterologous unheated cross-reactive antigens. These antisera are then used in slide agglutination assays with live bacteria. Over fifty serogroups have now been recognized in this scheme.

In the case of Campylobacter heat-labile antigens, those of C. fetus were the first to be examined (72). McCoy et al. (71) used agglutination and immobilization tests with antisera directed against intact flagella, the principle flagella antigen, the O antigen and a superficial "glycoprotein". Extraction of bacteria in glycine-hydrochloride buffer revealed three antigens, a, b, and c upon immunodiffusion and immunoelectrophoresis with whole cell antisera. These antigens were later determined to be superficial somatic antigens (73). Intact

flagella from a CsCl separation produced no reaction in immunodiffusion tests (71). However, solubilization of the flagella with acid released three antigens e, d, and c. Antigen e was present in the greatest abundance. Antigens c and d were found to be in the unsheathed flagellum, but their specific identity has not been determined.

In the case of *C. jejuni* and *C. coli*, Logan and Trust (64), using Western blotting techniques, showed that a 92.5 Kdal OM protein contributed to heat-labile antigenic specificity, and demonstrated that flagellin was immunogenic and appeared to carry both serospecific and cross-reactive common antigens (65). A 31 Kdal surface protein was also found to be a common heat-labile surface antigen. Subsequent studies by others have confirmed these observations (5,15,82). The contribution of flagella to serospecificity of some Lior serotypes was reported by Wenman *et al*. (113). These workers showed that aflagellated mutants were untypable with the heat-labile typing serum of Lio 6, 5, and 7, while the parent strains were typable. Thus it was apparent that flagellin was an essential heat-labile antigen used in the typing of Lio 5, 6, and 7 serogroups. Western blot experiments also showed that the most consistant immunogen in human infections was the flagellin. Western blot analysis of the human serum antibody response to *C. jejuni* cellular antigen preparations also showed bands corresponding to flagella, the major OM protein, a 43 K protein, and some LPS (79). This again supports the conclusion that the flagellum is a major surface antigen of *Campylobacter*. In certain cases the flagellum appears to be the serospecific determinant of the heat-labile Lior typing scheme. However, in other

serotypes a group of surface antigens may be involved in the scheme, including the flagellum and other OM proteins.

Flagella are also involved in the serotyping scheme of Hebert *et al.* (36). This serotyping scheme utilizes direct immunofluorescence and allows binding of antibodies to flagella to be visualized. Rabbits were immunized with formalinized whole cell of *C. jejuni*, *C. coli* or *C. fetus* cells. The immunoglobulin G fraction was then isolated from the antisera and fluorescein-labelled. These conjugates divided *C. jejuni* into 10 groups, *C. coli* into 2 groups, and C. *fetus* into 2 groups. While many other serotyping schemes have been developed using antisera against formalinized cells, live cells, or cells heated at 100°C, or a combination of these treatments they have not received general acceptance.

Flagella

Flagella are subcellular organelles responsible for bacterial motility, function in chemotaxis, and have been implicated in virulence. In most bacteria this organelle is composed of three distinct subunits, the basal body, the hook, and the filament.

The components of the basal body of Gram-negative organisms vary in MW from 9,000 to 60,000. These components form four ringlike structures bound to the central rod, ending in the hook structure (92). The outer ring of the basal body is bound to the LPS of the OM, the second ring is bound to the peptidoglycan layer, and the two inner rings are bound to the plasma membrane. The basal body, due to technical difficulties, is difficult to isolate and thus little is known about its molecular structure.

The hook, located at the base of the flagellum, is composed of a single polypeptide subunit of MW 42,000 in the case of E. coli and Salmonella. The hook protein is very different from the flagellum protein (92). As the hook represents only 1% of the total flagellar protein it is difficult to isolate and its function is unknown (1). However, it is thought to act as a joint at the base of the filament and to allow for circular motion to be transmitted to the flagellum. The proximal end of the structure terminates in the cell outer membrane at the basal structure and the distal point of the hook also acts as the initiation point for filament growth (43).

The flagella filament, located at the tip of the hook can be easily isolated by shaking and the free flagella can then be purified by differential centrifugation. Further purification may be obtained with salt or ethanol purification. Freezing and thawing and chromatography on diethylaminoethylcellulose columns can also be used in flagella purification (41). By adjusting the pH and ionic environment flagellin from solubilized filaments can be made to re-assemble into flagella filaments after other proteins have been removed by centrifugation (41).

Using electron microscopy the flagellum filament of Salmonella has been shown to be 4.5 nm in diameter (47). Cross-sectioning has also revealed a hollow center surrounded by five chains. Molecular models have been proposed for the arrangement of flagellin in flagella in which one, three, or five chains of flagellin molecules are helically wound and hexagonally packed. This arrangement is called Type A flagella (41). In Type B flagella, on the other hand, the flagellin is thought

to be packed in thick longitudinal lines.

Electron microscopy has also shown that the flagellar filament grows in a polar fashion by adding subunits to one end (3). Experiments using amino acid analogues with Salmonella (42) and pulse labelling experiments with B. subtilus (27) have shown that the amino acids are incorporated at the flagellar tip. Thus it is hypothesized that the subunits are synthesized inside the cell and then transported through a central cavity down the length of the flagellar filament and incorporated into the growing tip (92).

The molecular weight of flagellin was first estimated to be about 40,000 for Protease vulgaris, Bacillus subtilus, and Salmonella typhimurium (92). Subsequent studies have shown that MW's can vary between 40,000 and 65,000. Amino acid compositions of flagellins differ but have some common features. For example, both tryptophan and cysteine are absent, whereas aspartic acid, alanine, glutamic acid, threonine, glycine, leucine, valine, lysine, serine, isoleucine, argenine, tyrosine, phenylalanine, and methionine are present in all flagellins analyzed. The relative amounts of each amino acid decreases in approximately the order given above. The amino acids proline and histidine are present in small amounts or are not present at all (92).

Bacterial flagella by virtue of their surface exposure are antigenic. When motile cells react with antiserum to flagellar filaments, the filaments are cross-linked by the antibodies and the cells are immobilized (53). In tube tests this H-type agglutination is characterized by the formation of large flocculent particles which disperse by

shaking. No damage is done to the cells as motility resumes when the antibodies are destroyed. H-agglutination therefore contrasts with O agglutinations which form a small, compact agglutinate which is difficult to disperse.

The presence of flagella specific antigens was first recognized in *Salmonella*. Comparative amino acid analysis of flagellins of antigenically distinct *Salmonella* flagella showed that the antigenic difference was associated with the differences in amino acid composition between flagellin molecules (75). Other studies demonstrated that the specificity of a flagellar antigen was a reflection of the surface conformation of the flagellin molecule in the flagellin filament (47). This in turn was of course dependent on the amino acid sequence. It is also likely that the organization of flagellin in the flagellum accounts for some of the antigenic differences seen. For example, the interactions between several adjacent flagellins could give rise to determinants not found in the individual subunit (75).

It is also apparent that each filament has several antigenic determinants. For example, filaments from cells of *B. pumilus* consist of more than one protein and have more than one determinant (104). Within a filament, identical flagellins may exist in more than one conformation with different determinants exposed. Also, the two phases of *Salmonella* flagellin, although similar, have different antigenic determining groups. For example, 16 of 30 tryptic peptides of flagellins possessing the H antigens 1,2 are identical with those from flagellins possessing the i antigen although they are unrelated immunologically (74).

The immunogenic and antibody-binding characteristics of flagella are strongly influenced by flagellin comformation. Flagellin can undergo repeated spatial changes in response to environmental conditions such as pH and temperature. For example, at pH 2 flagellin will unfold and buried residues will become exposed thus changing the flagellin's antigenicity. Increases in temperature can also have the same effect.

Phase variation of flagella. Phase variation was discovered by Andrews in *Salmonella typhimurium* in 1922 (2). He discovered that mass cultures from a single clone of *Salmonella* would agglutinate with two different antisera directed against their flagellar filaments, anti-i or anti-1.2. A clone might react with i but on further subculturing would generate a derivative which reacted with 1.2. Stocker (103) then showed that the variation was a result of the ablility of the strains to switch from one flagella antigen (phase) to another with a probability that was two to three orders of magnitude higher than the frequency of mutation. This variation in phase occured at any time in the strains studied by Stocker. The frequency was between 10^{-3} to 10^{-5} per generation. Generally the frequency of transition in one direction is different than the frequency in the reverse direction.

Using genetic techniques, Lederberg and Iino (59) investigated the nature of this switch. From transduction experiments they were able to conclude that the structure of the flagellar filament was specified by two genes, H1 and H2. They also concluded that the ability to switch from the expression of one gene to the other was controlled by a genetic element linked to the H2 gene. Thus when the genes were active the H2

gene was phenotypically expressed and the H1 suppressed. Alternatively, when H2 was inactive, the H1 was expressed. It was also found that H1 and H2 were located far enough apart on the chromosome so as not to be co-transduced by phage particles (41). Thus, it was concluded that the switch involved a change at the DNA level and that the two loci were not closely linked to one another. As the two phases were at separate loci, phase variation could not be due to a mutation from one allele to another. Instead it must be the alternative expression of each of the two antigen genes present in the genotype.

Enomoto and Stocker (29) examined the properties of other genetic elements involved in regulating phase transition. The gene rh1 was found to control formation of a 16,000 MW repressor protein that repressed the synthesis of the H1 gene product. The expression of rh1 was associated with H2 gene expression. Another locus, ah2 had mutations that prevented H2 and rh1 expression. Mutations in ah2 only affected genes cis to the mutation. Thus, ah2 may be the promotor for the H2 and rh1 genes. Another cis acting element was hin (vh2). The wild-type hin^+ allowed phase variation at normal frequencies, while hin^- had two phenotypes, one was $H2^{on}$, in which only H2 was expressed and the frequency of transition to H1 expression was very low (10^{-6} -10^{-7} per bacterium per generation)(13). In the other phenotype, $H2^{off}$, only the H1 gene was expressed. These workers also concluded that phase switching likely involved a change at the DNA level, as hin only acted cis to the H2 gene, the gene could be transduced, and no H2 mRNA was found when H2 was off, this suggested that regulation was at

the transcriptional level. However the authors also indicated the regulation may involve other cytoplasmic factors. In later studies it was found that *hin* was a gene specifying a MW 19,000 cytoplasmic polypeptide which catalysed the inversion of the invertible region (55,106).

In subsequent studies the segment of *Salmonella* DNA which contained the genetic loci for phase variation was isolated using molecular cloning techniques (93,96,119). Heteroduplex analysis revealed an inversion adjacent to the H2 gene (119). Thus it was proposed that an inversion of this region causes phase variation in *Salmonella* flagella. Further work showed that the inversion region was approximately 800 base pairs, and inversion did not seem to depend on the *E. coli* Rec A recombination pathway (94). Gene fusion with phage suggested that the H2 gene promoter was included in this region (94,119). Using heteroduplex and restriction enzyme analysis, these 800 base pairs were shown to be capable of inversion and to contain specific sites at which recombination events occurred (118).

These "specific sites" for recombination were found by Zieg and Simon (120), who discovered that the inversion region was 995 base pairs in length and bound by a 14 base pair inverted repeat sequence (IRL and IRR). Thus, a homologous recombinational event between the 14 base pair inverted repeats would result in inversion of the DNA segment between them. The gene specifying the H2 flagellin synthesis was found to begin 16 base pairs outside the inversion region and within this region an open translational frame was found which could encode a low MW polypeptide (*ah*2). A summary of *Salmonella* phase variation is shown in

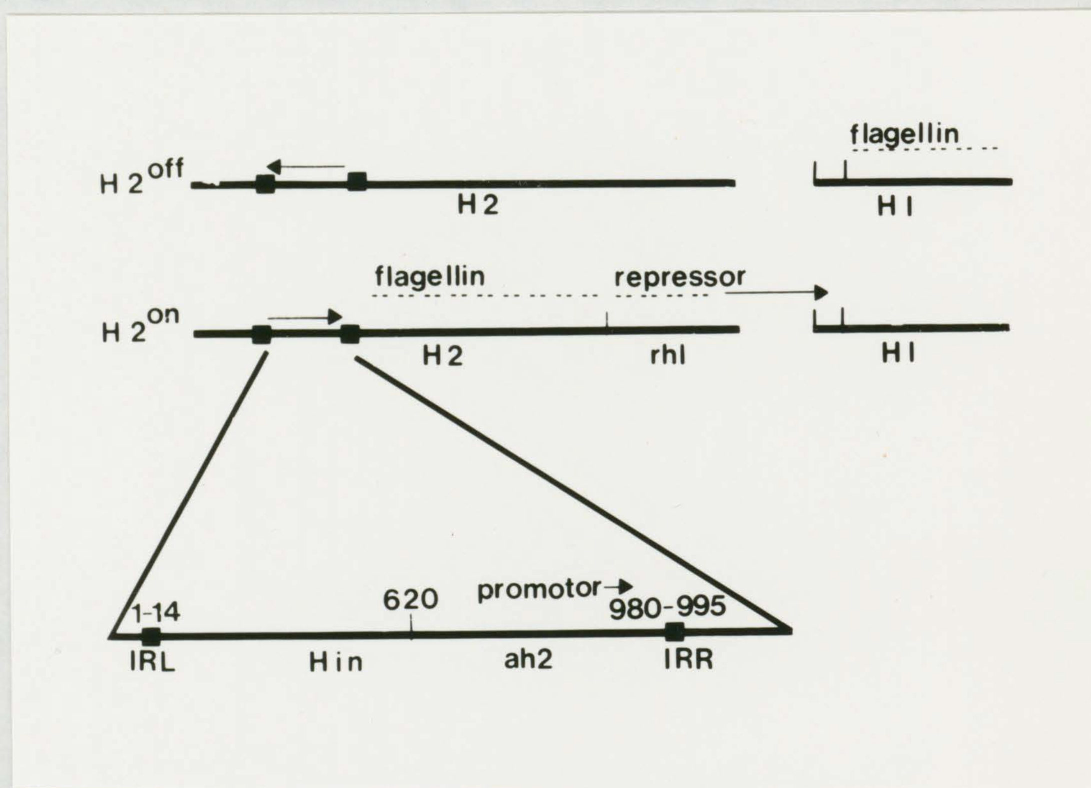
Figure 1.

Some investigators also believe that flagella phase variation may exist in Caulobacter cresentus (31,57,90,91) and Bacillus pumilus (101,104). However, little is known about these two systems and it is not yet known if the flagellins produced by these bacteria are a result of phase variation.

Role of flagella in Campylobacter colonization. There is evidence that the ability of Campylobacter to express flagella may be important in their ability to colonize the gastrointestinal tract. Morooka et al (78) examined C. jejuni colonization of the intestinal tract of suckling mice using oral challenge with a wild-type strain and several non-motile mutants. The wild-type strain colonized the intestinal tract two days after inoculation. Two non-motile strains lacking filaments and one non-motile strain with filaments were cleared from the intestine 2 days after challenge. One motile strain with a short filament colonized mice only when challenged with a large inoculum. These workers concluded that motility, and thus a working flagellum, was required by C. jejuni for effective colonization of the gut.

Newell and McBride (81) also investigated the biological properties of C. jejuni flagella using two variants from a motile clinical strain: a flagellated, non-motile variant and an aflagellate variant. Phenotypic and biochemical analysis of the strains and amino acid analysis of the flagella revealed the variants only differed from the wild-type in the absence of flagella or motility. The aflagellate strain colonized the intestine of mice poorly in contrast to the flagellated, non-motile

Figure 1. Diagram showing the components of the phase variation system for Salmonella flagella. The top lines illustrate phase transition. The boxes represent the invertible regions and arrows indicate the orientation of DNA sequences. The lower line represents a map of the invertible region. The numbers refer to nucleotide sequences (95).



strain and the wild-type strain which colonized the intestine readily. This suggested that with *C. jejuni*, flagella are necessary for colonization of the intestine. Lee *et al*. (60) have also found that human isolates of *C. jejuni* colonize the mucus on the outer surface and deep within the intestinal crypts of gnotobiotic or germ-free mice. To complete this colonization, flagella would be essential to the bacteria.

As *Campylobacter* flagella are highly antigenic, heat-labile proteins required for colinization of the intestine, the role of flagella in the Lior serotyping scheme was examined, with particular reference to Lio serotype 8.

MATERIALS AND METHODS

Bacterial strains and growth condition. *Campylobacter* strains examined in this study, and their sources, are listed in Table 1. Stock cultures were maintained at -80°C in 15% (v/v) glycerol-Trypticase soy broth (TSB, BBL Microbiology Systems; Cockeysville, Md.). For this study, cultures were grown at 37°C in an atmosphere containing 10% (v/v) CO_2 on chocolate blood agar plates (CBA) containing *Campylobacter* antibiotic selection solution (0.001% wt/v vancomycin, 0.0005% wt/v trimethoprim, 0.00075% wt/v colymycin). For large flagella preparations, cultures were grown on Mueller-Hinton agar (Difco Laboratories; Detroit, Mich.) in 150 mm Petri dishes.

SDS-PAGE. Components of the various preparations were separated by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel system described by Laemmli (56). Proteins were stacked in 4.5% acrylamide and separated in 7.5% acrylamide. On occasion, separating gels of 12.5%, and 15% were used. Gels were run at a constant voltage of 100 V per gel until the tracking dye entered the separating gel; the voltage was then increased to 200 V. Protein was normally visualized with Coomassie blue, although some gels were silver stained by the method of Wray *et al*. (116).

SDS-urea-PAGE. Peptides were separated by the SDS-urea-peptide gel system described by Swank and Munkres (105). Peptides were stacked in 6.2% acrylamide and separated in 12.5% acrylamide. Stacking and separation were done in the presence of 8 M urea and 0.1% SDS. Peptides were applied at 2 to 5 µg per lane. Gels were run at a constant current

Table 1. *Campylobacter* strains

	Strain	Source
C. jejuni	VC74	This laboratory, human feces
	VC83	Ex-NCTC[a] 11168, human feces
	VC84	H. Lior[b], human feces
	VC87	H. Lior, human feces
	VC90	H. Lior, human feces
	VC91	H. Lior, human feces
	VC150	H. Lior U.K., human feces
	VC154	H. Lior U.K., human feces
	VC155	H. Lior U.K., human feces
	VC156	H. Lior U.K., human feces
	VC159	H. Lior U.K., human feces
	VC162	H. Lior U.K., human feces
	VC163	H. Lior U.K., human feces
	VC168	H. Lior U.K., human feces
	VC185	This laboratory, human feces
C. coli	VC85	H. Lior U.K., human feces
	VC92	H. Lior U.K., human feces
	VC96	H. Lior U.K., human feces
	VC143	H.Lior, Bangladesh, human feces
	VC144	H.Lior, Bangladesh, human feces
	VC146	H.Lior, Bangladesh, human feces
	VC147	H.Lior, Bangladesh, human feces
	VC149	H.Lior, Bangladesh, human feces
	VC167	H.Lior, U.K., human feces
C. fetus	VC78	Ex-CIP[c] 5396, sheep fetus brain

[a] NCTC, National Collection of Type Cultures, London, United Kingdom.

[b] H. Lior, National Enteric Reference Centre, Ottawa, Canada.

[c] CIP, Collection de l'Institut Pasteur, Paris, France. Type species of the genus.

of 20 mA per gel. Peptides were fixed overnight in 25% isopropanol, 10% acetic acid and visualized with Coomassie blue.

Silver staining of LPS. LPS in whole cell lysates was determined by modification of the procedure of Hitchcock and Brown (38). Cells were boiled for 5 min in SDS-PAGE solubilization buffer then digested with proteinase K (Merck; Rahway, N.J.) at 60°C for 1 h at a ratio of 10 mg of cells to 1 mg of proteinase K. Samples were loaded onto SDS-PAGE to a maximum of 25 μg of cells per lane. After electrophoresis, gels were stained for LPS by the silver staining method of Tsai and Frasch (110).

Glycine extraction. Glycine extraction was performed by the method of McCoy *et al*. (70). Cultures were harvested into distilled water, washed twice and then suspended in 0.2 M glycine-hydrochloride, pH 2.2 (3 g cells per 100 ml). The suspension was stirred for 15 min at room temperature, and whole cells were removed by centrifugation at 12,000 x *g* for 15 min. The supernatant was neutralized with NaOH, dialyzed against distilled water, and lyophilized.

For small-scale rapid extractions (mini-glycine extraction), a 0.3 cm loopful of bacteria was suspended in 100 μl of 0.2 M glycine-hydrochloride, pH 2.2 and allowed to sit 5 min. Whole cells were removed by centrifugation at 12,000 x *g* in a MSE Microcentaur centrifuge.

Isolation of flagella. Cells were harvested in 20 to 30 ml of distilled water and homogenized twice for 30 s in a bead blender. Cells were pelleted by centrifugation at 10,000 x *g* for 1 h at 4°C, washed in distilled water, and removed by centrifugation at 10,000 x *g* for 20 min. The supernatants were pooled and centrifuged at 100,000 x *g* for 1

h at 4°C. The flagella-containing pellet was suspended in distilled water, adjusted to pH 2.0 with HCl and held at 0°C for 15 min to ensure complete flagellum disaggregation. Material insoluble at pH 2.0 was removed by centrifugation at 100,000 x g for 1 h, and the supernatant was adjusted to pH 7.0 with NaOH and left at 0°C for 30 min to allow flagella reaggregation. Flagella were concentrated by lyophilization or Amicon filtration (YM 30 filters with MW cut off > 30,000) for which the flagella solution was adjusted to pH 2.0 with trifluoro-acetic acid (TFA) and filtered.

High performance liquid chromatography of flagellin. A C8 column (RP-8 Spheri-10, Brownlee Labs; Santa Clara, CA) was used to purify flagellin and to separate peptides from flagellin trypsin digests. The Beckman HPLC system (Beckman Instruments Inc., Berkeley, C.A.) used in this chromatography consisted of two 100 A pumps, a model 421 controller, a model 352 injector, a model 165 detector and a Kipp and Zonen model BD41 chart recorder. The flagellin sample was centrifuged 5 min in an MSE Microcentaur centrifuge at 12,000 x g to remove insoluble material. 500 µl of the supernatant containing approximately 250 µg of protein was injected into the HPLC and eluted with a series of linear gradients of 0.1% TFA in water to 100% acetonitrile. The linear gradient system used for flagellin purification was as follows:

0-40% acetonitrile in 5 min

40-60% acetonitrile in 20 min

60-100% acetonitrile in 10 min

0% acetonitrile in 5 min

For separation of trypsin digests a gradient of 0-60% acetonitrile was used. The absorbance of the effluent was monitored at 230 and 280 nm.

Flagellin purification using ion exchange chromatography. To purify VC 167 phase 2 flagellin, ion exchange chromatography was used. The Beckman HPLC system previously employed was used in this separation, along with a mono-Q column (Pharmacia Fine Chemicals; Uppsala, Sweden). The flagellin sample was centrifuged 5 min in a MSE Microcentaur centrifuge at 12,000 x g to remove insoluble material. 500 µl of the supernatant containing approximately 250 µg of protein was injected into the HPLC and eluted as follows: the column was run isocratically for 10 min in 10 mM TRIS pH 4.0, and a gradient from 0-10% 1M NaCl in 10 mM TRIS pH 4.0 was then applied to the column for 5 min. The column was run isocratically until the protein was eluted. The absorbance of the effluent was monitored at 230 and 280 nm.

Bridge selection of cells producing flagella of different antigenic specificity. Cells producing flagella in antigenic phase 1 or 2 were grown 24h, and then streaked on one side of a CBA plate from which a strip of agar had been cut out of the center. A sterile filter paper was placed across the center well to form a bridge. 25 µl of a 1:20 dilution of antiserum was applied to the bridge. When growth appeared on the other side of the bridge, mini-glycine extracts were prepared and flagellin MW determined by 7.5% SDS-PAGE.

Rate of flagella phase switching. The thioglycollate medium of Caldwell *et al*. (17) was used to determine the rate of flagella phase switching. Thioglycollate media (BBL Microbiology Systems; Cockeysville, M.D.)

containing 0.33% (wt/v) agar was prepared and kept warm. A single colony of bacteria producing phase 1 or phase 2 flagella was diluted 10^{-6} in this agar. Absorbed antiserum to phase 1 or 2 flagella was then added to the warm agar at a 1:500 dilution and plates were poured. Controls with no added antiserum were also poured. After two days the rate of flagella-phase alteration was determined by counting the number of motile and non-motile colonies in the antiserum-containing agar. The controls were also examined to determine the frequency of production of non-motile variants. Non-motile and motile colonies from both types of agar were then selected and cultured, and the MW of flagellin was determined by SDS-PAGE of mini-glycine extractions.

Amino acid composition. 20 nmoles of protein was dissolved in 1 ml of constant boiling HCL and placed in a necked tube. The tube was sealed under vacuum, held at 110°C for 18 h then opened. The sample was evaporated to dryness in a desiccator and resuspended in 200 μl of 0.07 M sodium citrate pH 2.0. 100 μl of sample was applied to a Beckman 119 GL amino acid analyzer.

Cysteine recovery. The cysteine composition of the protein was determined by the method of Hirs (37) as follows: 0.5 ml of H_2O_2 and 9.5 ml of formic acid were held at 25°C for 120 min to form performic acid. The sample was dissolved in 100 μl of formic acid and 20 μl of methanol, then chilled to -15°C. 200 μl of performic acid was then added and the reaction was allowed to proceed at -15°C for 120 min. Cold HPLC grade water (25 ml) was added to the sample and lyophilized. The lyophilized material was hydrolyzed with HCL as previously

described and applied to a Beckman 119 GL amino acid analyzer.

Tryptophan recovery. The tryptophan composition of the protein was determined by the method of Penke et al (83). 50 μg of the protein was hydrolyzed with 100 μl of 3 N mercaptoethanesulfonic acid at 110°C for 24 h under vacuum. 100 μl of 1 N NaOH was added and the sample was analyzed for tryptophan in a Beckman 119 GL amino acid analyzer.

N-terminal amino acid sequence analysis. Sequencing was carried out on an Applied Biosystems Model 470A sequencer (Applied Biosystems; Foster City CA). HPLC purified flagellin (50 nmoles) was applied to the sequencer and analyzed using a standard Applied Biosystems program.

Production of antisera.

Antiserum directed against purified phase 1 flagellin: An adult New Zealand white rabbit was immunized with phase 1 flagellin from *C. coli* VC 167. 600 μg of flagellin was emulsified with 900 μl of Freund's complete adjuvant and injected subcutaneously into the back and the hind legs. A booster injection was given in a similar manner in Freund's incomplete adjuvant 21 days after the initial immunization. On day 35 the rabbit was bled, and the sera was collected and stored at -20°C in 200 ul portions. Prebleed sera was taken and used as control sera.

Antiserum directed against live bacteria producing phase 1 or 2 flagella: Two adult New Zealand white rabbits were immunized by the method of Penner (84) with live bacteria producing either phase 1 or phase 2 flagella. The confluent bacterial growth from 2 CBA plates was suspended in saline to an A_{625} of 0.375. The bacteria were injected intravenously five times in a 2 week period with 1,2,2,4, and 4 ml of

the suspension. On day 48 the rabbits were bled and the sera was collected and stored at -20°C in 200 ul portions. Prebleed sera was taken and used as control sera.

Absorption. Antisera were absorbed with a homologous heat-stable antigen suspension. Cells from two plates was suspended in 5 ml of phosphate-buffered saline and heated at 100°C for 2 h. Bacterial cells were collected by centrifugation at 12,000 x *g*, mixed with equal volumes of sera, and incubated at 10°C for 1 h. Antibodies to heat-stable antigens were removed by centrifugation at 12,000 x *g*. Antisera were also absorbed with a live cell suspension of bacteria producing flagella in the alternative phase to which the rabbit sera was produced.

Immuno-electron microscopy. Two ml of saline was added to the surface of a CBA culture plate and cells were suspended by gentle aggitation. A drop of suspension was removed and a formvar coated grid was placed on the drop for approximately 1 min. After washing three times in saline, the grid was placed on a drop of antiserum and incubated for 30 min at 37°C in a moist environment. Unbound antiserum was removed by washing 3 times in distilled water, and the grid was negatively stained with 1% uranyl acetate (pH 4.2). For better visualization of bound antibodies, 2 µl of protein A gold conjugate (Janssen Life Science Products; Beerse, Belgium) was applied to the grids after antiserum application, and incubated at 37°C for 30 min in a moist environment. The grids were then stained with uranyl acetate. Grids were examined in a Phillips EM 300 electron microscope.

Immunoblotting. After SDS-PAGE, separated components were transferred

from the slab gel to nitrocellulose paper by the methanol-TRIS glycine system described by Towbin et al (109). Electroblotting was carried out in a transblot apparatus (Bio-Rad Labs; Richmond, CA.) overnight at 60 V. After blocking unreacted sites for 2 h in a 1% (wt/v) solution of gelatin in 10 mM TRIS (pH 7.4)-0.9% (wt/v) NaCl, the nitrocellulose paper was incubated for 2 h with antiserum. After washing, the sheets were incubated with ^{125}I-labelled staphylococcus protein A (5×10^5 CPM/ml) for 2 h. Detection of bound radiolabeled protein A was accomplished by autoradiography of washed, dried sheets using intensifying screens in a Kodak Xomatic cassette (Kodak; Rochester, N.Y.) and XAR-5 X-ray film (Kodak; Rochester, N.J.). The film was exposed for 1 to 4 days.

Iodination of glycine extracts, flagella and protein A. Proteins were ^{125}I-labelled using a modified Hunter and Greenwood chloramine T procedure (39). 50 µg of protein in 0.5 M phosphate buffer (pH 7.4) was reacted with chloramine T in buffer and 1 mCi of ^{125}I for 3 min. The reaction was stopped with 4 mg/ml sodium metabisulphite and 10 mg/ml potassium iodide and 5% gelatin in PBS. The reaction mixture was then dialyzed overnight to remove unbound ^{125}I.

Slide agglutinations. The ablity of antisera diluted 1:16 in PBS to agglutinate live cells of Campylobacter was tested on glass slides by the method of Lior (63). Presence or lack of agglutination was determined after 60 s.

Enzyme-linked immunosorbent assay (ELISA). The ELISA procedure was essentially that of Engvall and Perlmann (28). Antigens were tested at

0.5 to 2 μg per well, in triplicate, and the developing antibody was alkaline phosphatase to either conjugated goat anti-mouse or anti-rabbit immunoglobulin (Sigma Chemical Co.; St. Louis, Mo.).

Immunofluorescence of living cells (IFAT). Cells were suspended in PBS at 4°C. 20 μl of a cell suspension (10^9 cells/ml) was then added to 50 μl of antibody and diluted to a final concentration of 1:500 in PBS. The reaction mixture was incubated for 60 min at 4°C. The cells were washed three times by centrifugation at 2000 x *g* in a Beckman TJ61 centrifuge. 20 μl of fluorescein goat anti-rabbit globulin conjugate (Gibco Laboratories, Grand Island, N.Y.) at a 1:80 dilution in PBS was then added and incubated at 4°C for 1 h. Excess antibody was removed by washing three times as above, and pellets were suspended in PBS. The cell suspension was applied to a slide and observed immediately by epifluorescence on a Ziess fluorescence microscope fitted with an epifluorescence attachment.

Radio-immunoprecipitation (RIP). The immunoprecipitation method used was that described by Dooley *et al*. (25). Briefly, ^{125}I-labelled samples were diluted in immunoprecipitation buffer to 10,000 CPM/100 μl and placed into a 10 ml conical tube. Antibody was added at a predetermined dilution and incubated 15 min at room temperature. A 50% (wt/v) suspension (in immunoprecipitation buffer) of protein A-Sepharose 4B (Pharmacia; Uppsala, Sweden) was then added to the tube and incubated 15 min at room temperature. The solution was diluted to 10 ml in buffer, spun at 15,000 x *g* for 15 min in a Beckman TJ61 centrifuge, and the supernatant was removed. The wash was repeated twice. The pellet was

resuspended in a small volume of buffer and placed in a microcolumn (25). The column was dried by centrifugation and 20 µl of SDS-PAGE solublization buffer was added and heated to 60°C for 5 min. The sample was centrifuged into a fresh tube and applied to SDS-PAGE. Detection of immunoprecipitated protein was visualized by autoradiography of dried slab gels.

Enzyme digestions of flagella. Chymotrypsin, (50 units/mg TLCK treated, Sigma Chemical Co.; St. Louis, M.O.), trypsin (15,000 BAEE units/mg TPCK treated, Sigma), and proteinase 6 (4.4 units/mg, Sigma) were prepared in 10 mM TRIS pH 7.8. Enzymes were added to phase 1 or phase 2 purified flagellin suspended in 10 mM TRIS pH 7.8 at dilutions of 1:100, 1:200 and 1:400 (wt/wt). The reactions were allowed to continue for 30 min and then repeated for 1 h at 25°C. Trypsin and chymotrypsin activities were stopped by the addition of an equivalent amount of trypsin inhibitor (10,000 BAEE units/mg, Sigma). Proteinase 6 activity was stopped with the addition of a saturated solution of phenylmethylsulfonyl floride (PMSF) at 1% (wt/wt, Sigma).

Cyanogen bromide digestion of flagella. Cyanogen bromide dissolved in formic acid was added to the preweighed flagella sample at 200 times the flagella methionine molarity. The reaction was allowed to proceed at room temperature overnight. Following incubation, the digestion mixture was diluted 25 fold in distilled H_2O and concentrated by lyophilization.

RESULTS

At the outset of this project, the objective of the study was to evaluate the role of flagella in the Lio 8 serogroup of the Lior serotyping scheme. This serogroup contains both C. jejuni and C. coli strains, and is one of the most common Lior serogroups isolated from humans. Some 18 strains belonging to Lio 8 were avalaible in the University of Victoria Campylobacter culture collection. Lior typing antisera were graciously provided by Hermy Lior, Enteric Reference Center, Laboratory Center for Disease Control, Ottawa.

Slide agglutinations. To confirm that all putative Lio 8 strains indeed belonged to the Lio 8 serogroup, slide agglutinations were carried out at the 1:16 dilution recommended by Lior (63). All strains previously labelled Lio 8 gave rapid cell agglutination (Table 2). Non-serotype 8 strains included as controls were not agglutinated by the antiserum.

IFAT analysis of serogroup Lio 8. IFAT's of live Campylobacter cells were carried out to confirm that the epitopes recognized by Lio 8 were surface exposed. The immunofluorescence assays were carried out with a 1:500 dilution of Lio 8 antiserum. This dilution was chosen in an effort to minimize binding of lower titre cross-reactive antibodies. The results are shown in Table 2. The Lio 8 antiserum reacted with strains from both 8 and 29 serotypes. This cross-reactivity between certain strains belonging to 8 and 29 serotypes has been observed previously (Lior, personal communications). The fluorescence observed with Lio 8 strains appeared as small dots. By bright field microscopy the cells appeared to be immobilized, suggesting that the antibodies

Table 2. Agglutination and IFAT reactions of Campylobacter Lio 8 strains.

Strain	Antiserum Lio 8		Antiserum Lio 29
	Agglutination	IFAT	IFAT
Serotype Lio 8			
143	+	wc[b]	wc
144	+	+[c]	-
145	+	+	-
146	+	+	-
147	+	+	-
149	+	+	-
150	+	+	-
155	+	+	-
156	+	+	-
159	+	+	-
167	+	+	-
Serotype Lio 29			
148	nt[a]	wc	wc
157	nt	wc	wc
158	nt	wc	wc
161	nt	wc	wc
168	+	wc	wc
Other Serotypes			
74	-	-	-
81	-	nt	nt
83	-	nt	nt
84	-	nt	nt

+ is a positive reaction, - is a negative reaction, [a] is not tested, [b] is fluorescence as whole cells, [c] is fluorescence as small dots

were bound to flagella. The fluorescence observed with Lio 29 cells was different. In this case the whole cell fluoresced. Thus it appeared that the Lio 8 antiserum also contained antibodies directed against cell surface epitopes of Lio 29 cells while at the 1:500 antiserum dilution the Lio 8 antiserum recognized flagella on serogroup 8 cells.

As Lior 29 strains were cross-reactive with Lio 8 antiserum, IFAT assays were also performed on strains belonging to both serotypes using Lio serotype 29 antiserum. The results are also shown in Table 2. From these results it was apparent that this antiserum was directed against antigens on the surface of the Lio 29 cells and not the flagella and that 29 antiserum was not cross-reactive with Lio 8 strains.

Immunoblotting with Lio 8 and Lio 29 Antisera. Agglutination and IFAT results suggested that flagella may carry serospecific surface-exposed epitopes in the case of Lio 8 strains. To confirm that flagellar proteins were able to react with Lio 8 antiserum, immunoblotting was performed. This technique involves the separation of antigens by SDS-PAGE, followed by electrophoretic transfer of the antigens to nitrocellulose. The nitrocellulose bound antigens are then reacted with antibody followed by ^{125}I-labelled protein A and the antibody-antigen reaction is visualized by autoradiography. The ability of antibodies to react with linear epitopes in this procedure allows polypeptides carrying these epitopes to be identified. Immunoblots of whole cell lysates of VC strains 143, 144, 155, 156, 157 and 167 with 1:100 and 1:500 dilutions of Lior 8 and 29 antisera were performed. No reaction was obtained with the Lio 8 antiserum (results not shown). This

indicated that the epitopes recognized by this polyclonal antiserum were conformational and this conformation was destroyed by SDS-treatment. Similarly, no reaction was detected with Lio 29 antiserum again indicating that this antiserum was directed against conformational epitopes.

Immunoprecipitation with Lio 8 antiserum. An immunological detection procedure which allows molecules carrying conformational epitopes to be detected is radio-immunoprecipitation (RIP). This technique was therefore applied to the glycine extractable protein fraction of the type strain of serogroup Lio 8, VC 167. This fraction was chosen because other studies (65) had shown that it contained flagella and other surface proteins of the thermophilic Campylobacter cell. Therefore, when radiolabelled glycine extracts of type strain VC 167 were immunoprecipitated with Lio 8 antiserum at dilutions of 1:100, and 1:1000, autoradiography showed that only one protein was precipitated (Fig. 2, lanes 1, 2). This protein had a MW of 61,000 which corresponded to the MW previously reported for Campylobacter flagellin. No detectable proteins were precipitated by an unrelated antiserum (Fig. 2, lane 3) or from the glycine extractable protein fraction of an unrelated strain, VC 74 (Fig. 2, lane 4).

To confirm that the precipitated VC 167 protein was indeed flagellin, purified VC 167 flagellin was immunoprecipitated with Lio 8 antiserum. As can be seen in Fig. 3A, lane 4, VC 167 flagellin was immunoprecipitated. Importantly the MW corresponded to that of the protein immunoprecipitated from the glycine extracts. This indicated that the

Figure 2. Autoradiogram of SDS-PAGE profiles of proteins from VC 167 glycine extractable protein fraction immunoprecipitated by Lio 8 antiserum at various dilutions. Lane 1, 1:100 dilution; lane 2, 1:1000 dilution; lane 3, 1:100 dilution of Lio 29 antiserum; lane 4, VC 74 glycine extract precipitated with Lio 8 antiserum at a 1:100 dilution.; lane 5, ^{125}I-labelled VC 167 glycine extract. MW X 1000 on right. Exposure 24 h.

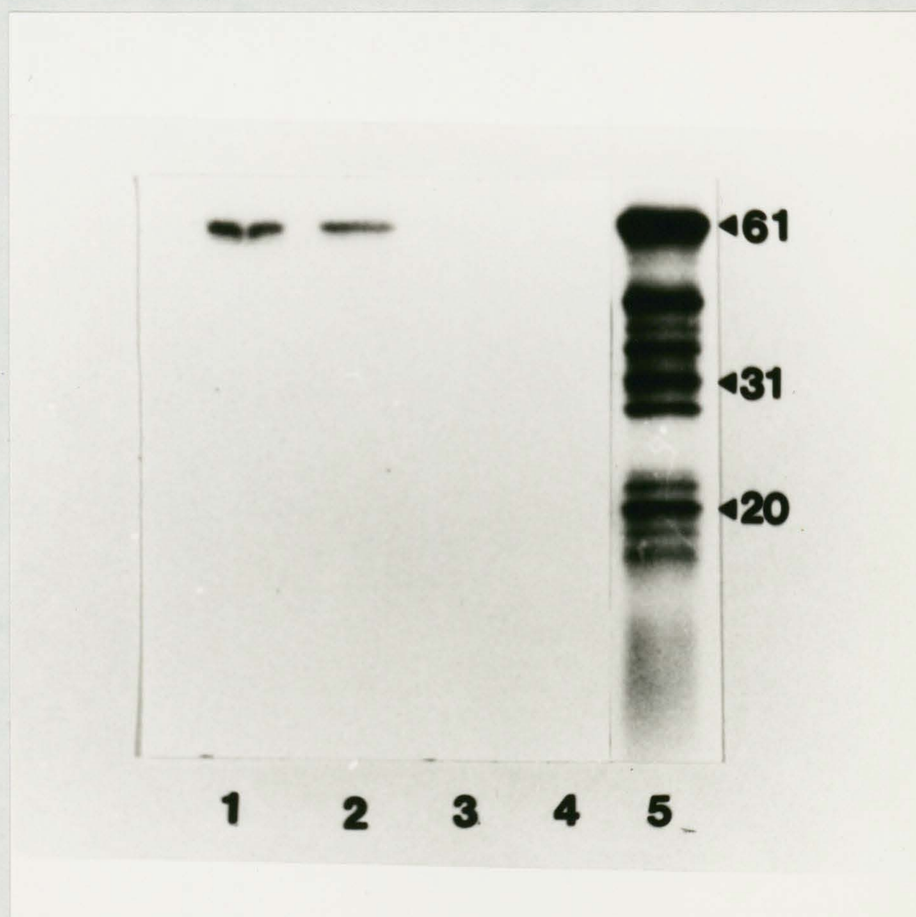
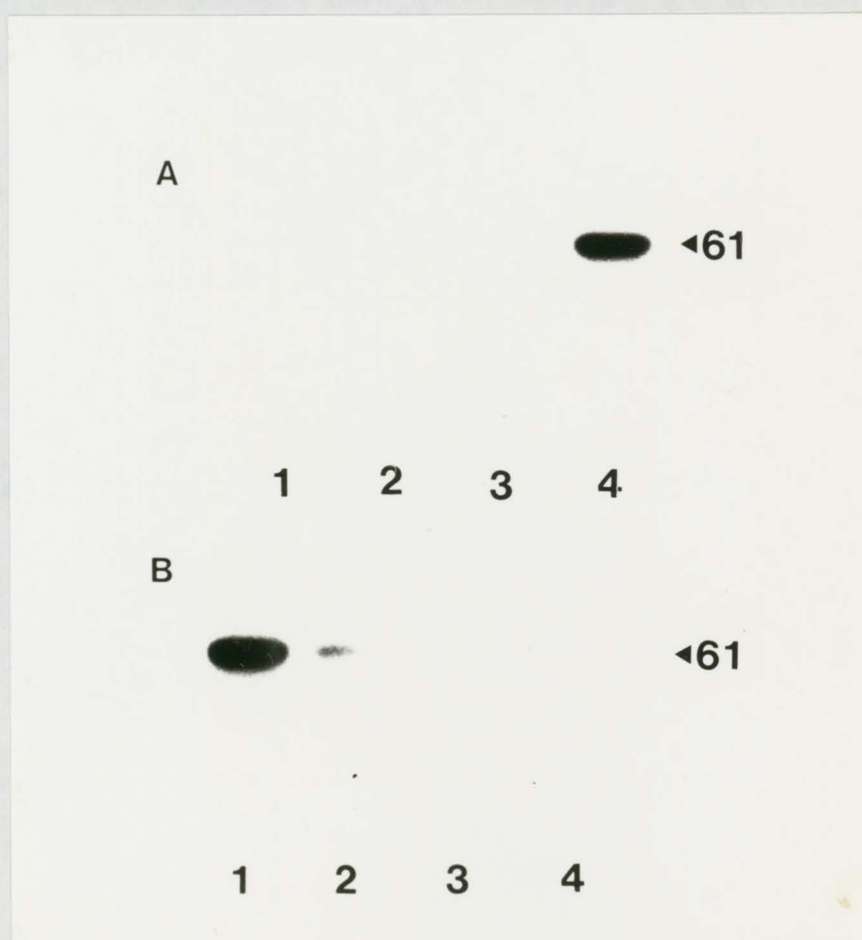


Figure 3. Autoradiograms of SDS-PAGE profiles of flagellin from Campylobacter strains immunoprecipitated with Lio antisera at a dilution of 1:100. Autoradiogram A: lane 1, VC 167 flagellin RIPed with Lio 29 antiserum; lane 2, VC 78 flagellin RIPed with Lio 8 antiserum; lane 3, VC 74 flagellin RIPed with Lio 8 antiserum; lane 4, VC 167 flagellin RIPed with Lio 8 antiserum. Autoradiogram B: RIP with Lio 8 antiserum and flagellin from strains; lane 1, VC 156; lane 2, VC 159; lane 3, VC 143; lane 4, VC 144. MW X 1000 on right. Exposure 24 h.



major epitopes to which the antibodies in the Lio 8 antiserum were directed were on the flagellin. The specificity of this reaction with Lio 8 flagellin was shown by the inability of Lio 8 antiserum to immune-precipitate flagellin from strains belonging to Lio 1, 4, 6, 11, 13, and 29 and from one strain of C. fetus (Fig. 3A, lane 2, and Table 3) and the inability of control Lio 29 antiserum to immunoprecipitate VC 167 flagellin (Fig. 3A, lane 1).

Flagellin and glycine extracts from four other Lio 8 serotype strains as well as several other serotype 8 strains were then immuno-precipitated with the Lio 8 antiserum to determine if these flagellins had the same epitopes as the type strain (Table 3). Flagellin from VC 156 and VC 159, both Lio 8 serotypes was immunoprecipitated (Fig. 3B, lanes 1 and 2). However flagellin from two other serotype 8 strains, VC 143 and VC 144, was not immunoprecipitated (Fig. 3B, lanes 3 and 4). These immunoprecipitations were repeated with fresh cultures several times with the same results. However, slide agglutination tests with these strains showed that they were still serotype Lio 8. This indic-ated that at the dilutions of antiserum used in the Lio 8 serotyping scheme (1:16), epitopes other than those on the flagella were involved in the typing scheme. IFATs at 1:16 dilutions provided further evidence of this as there was much staining of the cell surface.

SDS-PAGE flagellin analysis. The ability of Lio 8 antiserum to RIP flagellin from some but not all Lio 8 strains suggested that these strains were producing different flagellins. To investigate this pos-sibility, flagellin subunit MW was re-examined by SDS-PAGE using 7.5%

Table 3. RIP of purified flagellin and flagellin from the glycine extractable fraction (ge) of Campylobacter strains with Lio 8 antiserum at dilutions of 1:100, and 1:1000. A positive result was the appearance in SDS-PAGE of a single band of MW 61,000. No other proteins were immunoprecipitated.

Lio serotype	Flagellin from strain	RIP
C. fetus	VC 78	-
1	VC 87	-
4	VC 83	-
6	VC 84	-
11	VC 74	-
11	VC 91	-
13	VC 96	-
29	VC 168	-
8	VC 143(ge)	-
8	VC 144(ge)	-
8	VC 156(ge)	+
8	VC 159(ge)	+
8	VC 167(ge)	+

acrylamide rather than the 12.5% acrylamide previously employed. This change was made to allow for better separation of subunits in the MW range 50-70,000. When glycine extractable proteins from serotype 8 strains were examined, several strains were found to produce two flagellins of MW 57,000 and 61,000. Some Lio 8 strains appeared to produce only the MW 61,000 flagellin, while others produced only the 57,000 flagellin (Fig. 4A).

Subculturing of Lio 8 serotype strains. One explanation for the presence of two flagellins of different MW was that the *Campylobacter* cultures being glycine extracted contained a population of cells producing flagella composed of flagellin monomers of one MW and another population producing flagellin monomers of a different MW and that flagellin production was subjected to random switching at a quite high relative frequency. As Lior recommended subculturing strains several times before serogrouping to "stabilize" antigenic specificity, serial subculturing was performed on several serogroup 8 strains to determine the effect on the flagellin being produced. Mini-glycine extracts were prepared from a single clone from each subculture and subjected to 7.5% acrylamide SDS-PAGE. A summary of the results is shown in Table 4. Fig. 4B shows an example of one SDS-PAGE gel of these glycine extracts.

Some of the strains appeared to be producing only a 57,000 MW flagellin in detectable quantities, even after ten subcultures. For example, the two Lio 8 strains which did not react by immuno-precipitation, strains VC 143 and VC 144, produced only a low MW flagellin. The three Lio 8 serotypes which did immuno-precipitate (VC 156, VC 159 and VC 167)

Figure 4. SDS-PAGE (7.5%) of glycine extractable proteins of Lio 8 Campylobacter strains, stained by Coomassie blue. Gel A: st=MW standards, MW X 1000 on left; lane 1, VC 143; lane 2, VC 144; lane 3, VC 146; lane 4, VC 147; lane 5, VC 149; lane 6, VC 154; lane 7, VC 155; lane 8, VC 156; lane 9, VC 159; lane 10, VC 162; lane 11, VC 163; lane 12, VC 167. Gel B: lane 1, VC 162 1 subculture; lane 2, VC 162 10 subcultures; lane 3, VC 154 1 subculture; lane 4, VC 154 10 subcultures; lane 5, VC 155 1 subculture; lane 6, VC 155 10 subcultures; lane 7, VC 155 1 subculture; lane 8, VC 155 10 subcultures.

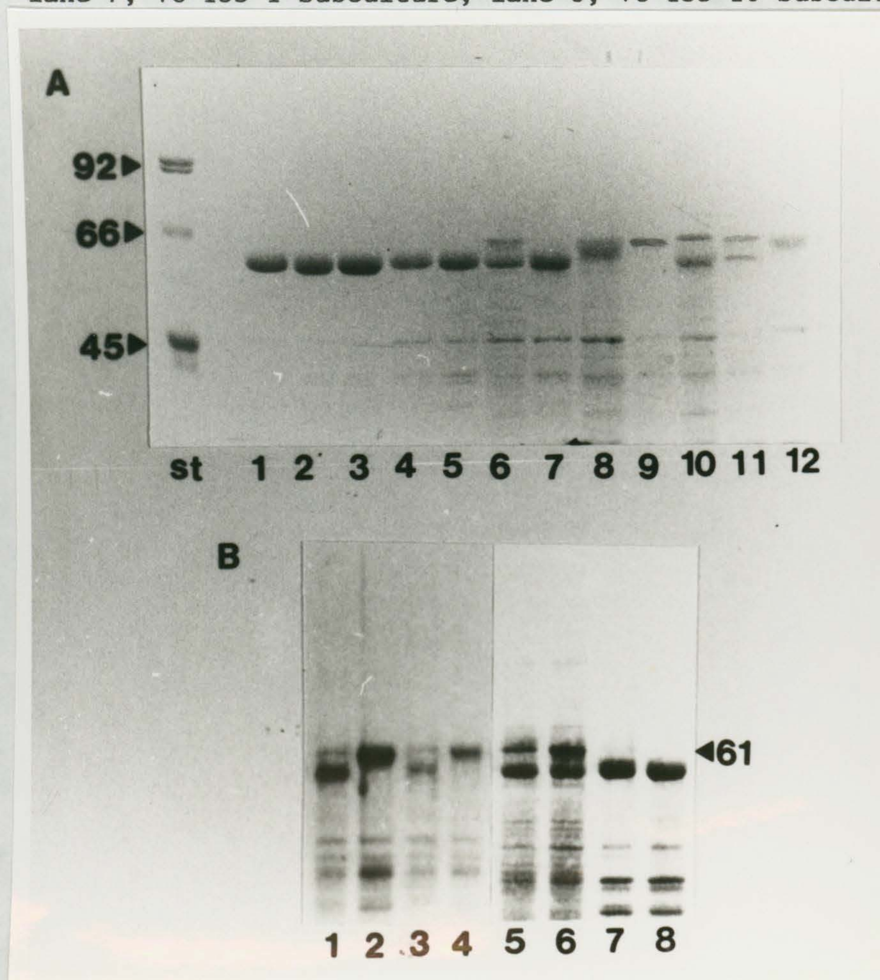


Table 4. Effect of subculture on flagellin MW. Glycine extractable proteins of Lio 8 strains were examined by 7.5% SDS-PAGE.

Strain	Subculture	Flagellin MW (x1000)
143	1	57
	10	57
144	1	57
	10	57
	1	57
	10	57
146	1	57,61
	10	61
155	1	57
	10	57
	1	57,61
	10	57,61
156	1	61
	12	61
159	1	61
	12	61
162	1	57,61
	7	57
	1	57
	7	57
147	1	57
	10	57
149	1	57
	10	57
150	1	57
	10	57
154	1	57,61
	10	61
167	1	61
	10	61
163	1	57,61
	10	57,61

continually produced flagellins of MW 61,000 in detectable quantities.

Other Lio 8 strains produced both flagellin types, and in some cases the lower MW flagellin could be eliminated on subculturing. Strains VC 146, VC 154, VC 155, VC 162, and VC 163 all produced both flagellins. However, strains VC 162 and VC 154 were observed to lose their lower MW flagellin molecule after subculturing (Fig. 4B, lanes 1,2,3 and 4). In one experiment VC 155 did not lose either flagellin molecule after subculturing (Fig. 4B, lanes 5 and 6), while in another experiment, with cells taken from the same stock culture, only the lower MW molecule was produced (Fig 4B, lanes 7 and 8). With VC 163, subculturing did not effect flagellin production. From these series of experiments it was apparent that a great deal of variability existed in flagellin production with respect to flagellin subunit MW. These results suggested that phase variation of flagellin production may be occurring in the strains being examined.

Immuno-electron microscopy. As SDS-PAGE showed evidence of two flagellins being produced by a given strain, some strains were examined by immuno-electron microscopy using Lio 8 antiserum to determine if two antigenically distinct flagella filaments were being produced by a given culture. Strains 143 and 144 which produced the MW 57,000 flagellin, which was not immunoprecipitated by Lio 8 antiserum, exibited flagella which did not appear to bind Lio 8 antibodies (Fig, 5A). Flagellin from strains 156, 159, and 167 were immunoprecipitated by Lio 8, had a MW of 61,000, and the flagella produced by these strains were coated by Lio 8 antibodies (Fig. 5B,5C). Upon careful examination of the grids, these

strains were also found to produce some flagella which were not coated by the antiserum (Fig. 5B,5C). As a control, C. jejuni VC 74 was reacted with Lio 8 antiserum and no flagella coating was observed. The immuno-electron microscopy also confirmed that Lio 8 strains 143 and 144 produced flagella which did not react with Lio 8 antiserum providing further evidence that the epitopes responsible for serogrouping in this serotype were not flagella-borne.

Separation of cells producing lower MW flagellin. To show that a strain could be switched from the production of one flagellin antigenic type, Lio 8, to the production of a different flagellin antigenic type, cells were selected which exhibited motility in the presence of Lio 8 antiserum. This was accomplished by a bridging technique. Lio 8 antiserum was placed on a sterile filter paper connecting two separate culture media surfaces. Bacteria from a single clone that crossed over the bridge were repassaged until SDS-PAGE analysis of glycine extracts showed only a single flagellin band with a MW different from that of the parent. The three strains (VC 167, 156, and 159) which consistantly produced the higher MW Lio 8 reactive flagellin (phase 1) were used in this experiment. After seven bridge transfers each strain was shown to produce the lower MW molecule (phase 2). A gel of the glycine extractable proteins of the three strains before and after "crossing the bridge" is shown in Fig. 6. The three VC strains producing the MW 61,000 flagellin (167 P1, 156 P1, 159 P1) were now producing the MW 57,000 flagellin (167 P2, 156 P2, 159 P2). Control transfers with VC 167 P1 in the absence of antiserum did not result in the lower MW

Figure 5. Electron micrograph of VC 144 (A), VC 156 (B) and VC 167 flagella (C) reacted with Lio 8 antiserum and negatively stained with uranyl acetate. Micrographs A and B were also reacted with protein A-gold for better visualization of antibody binding. The small bar in the lower right hand corner of each picture represents 100nm.

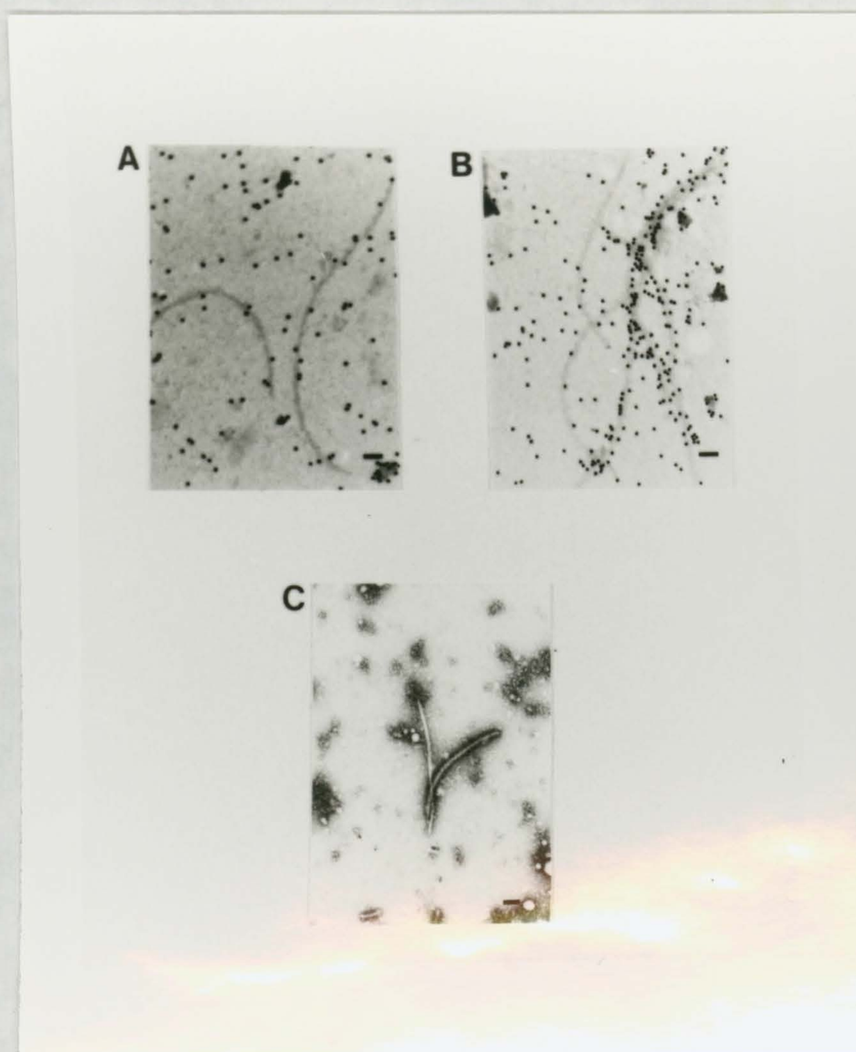
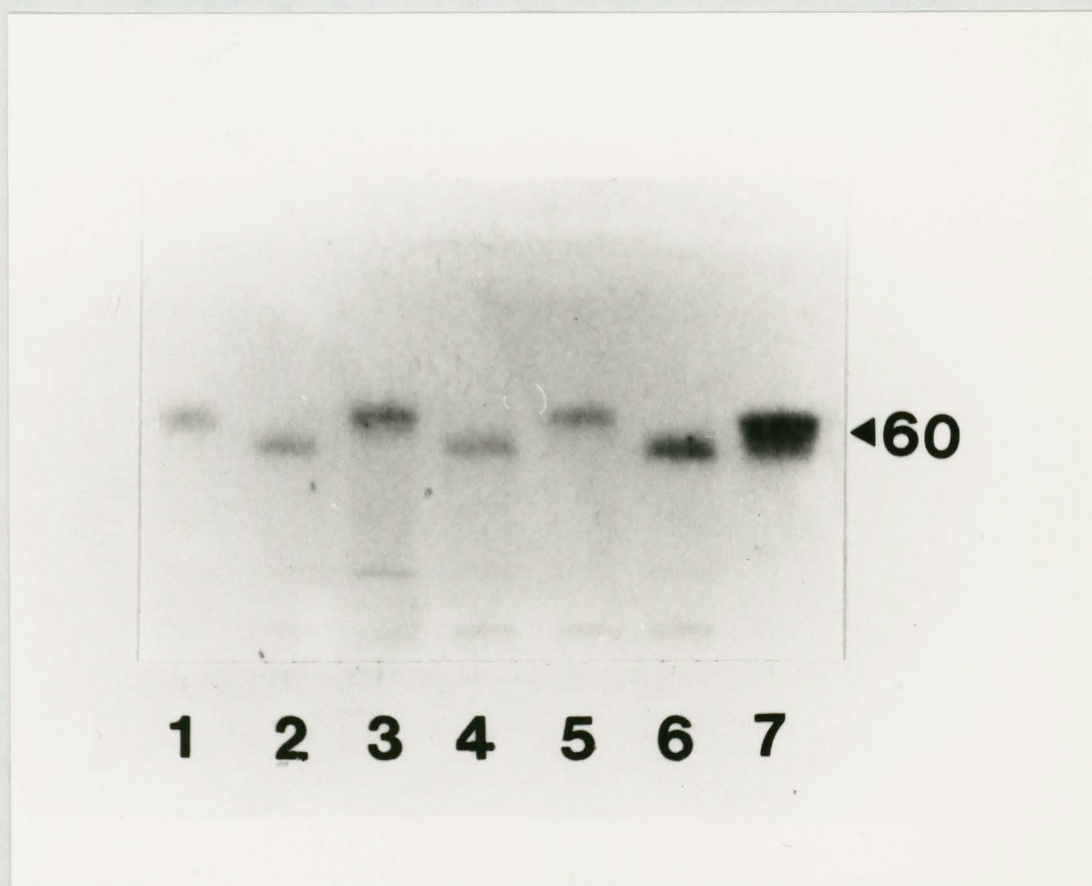


Figure 6. SDS-PAGE (7.5%) of 5 µl of glycine extractable proteins of <u>Campylobacter</u> strains before and after bridge transfer. Staining was with Coomassie blue. Lane 1, VC 156 P1; lane 2, VC 156 P2; lane 3, VC 159 P1; lane 4, VC 159 P2; lane 5, VC 167 P1; lane 6, VC 167 P2; lane 7, VC 167 P1 transferred in the absence of antiserum. MW X 1000 on right.



flagellin (Fig 6, lane 7).

The stability of this switch was then determined for the three strains by serial subculture. After 10 subcultures of strains VC 156 P2 and VC 167 P2 in the absence of selective pressure, the lower MW flagellin was always retained (Fig. 7). However in one set of subcultures of VC 159 P2, two flagellins were observed (Fig. 7, lane 4). In all other subculturing experiments only the phase 2 flagellin was observed. From these results it was concluded that the switching from phase 1 to phase 2 flagellin production was stable, with the possible exception of strain VC 159.

Preparation of antiserum to phase 2 flagellin. To extend the results obtained with the Lio 8 antiserum, polyclonal antiserum was prepared against VC 167 P2 live motile cells (LAH 2 antiserum). To increase the specificity of this antiserum it was absorbed against homologous heat stable antigens and motile live cells of VC 167 P1.

Separation of cells producing phase 1 and 2 flagellins. LAH 2 antiserum was then used in the bridging technique to isolate cells producing the higher MW phase 1 flagellin from cells producing the lower MW protein. Bacteria from a single clone of VC 167 P2 were serially passed over antiserum soaked bridges until SDS-PAGE analysis detected only the higher MW flagellin. As this antiserum was highly specific it only required two crosses to obtain cells producing phase 1 flagellin. This was repeated several times. The results in Figure 8A show that the VC 167 P2 cells (Fig. 8A, lane 2) had switched to producing the higher MW 61,000 flagellin (Fig. 8A, lane 3). After subculturing seven times

Figure 7. SDS-PAGE (7.5%) of 5 μl of glycine extractable proteins of VC Campylobacter strains immediately after phase transfer and after seven subcultures. Lane 1, 156 P2; lane 2, subcultured 156 P2; lane 3, 159 P2; lane 4, subcultured 159 P2; lane 5, 167 P2; lane 6, subcultured 167 P2. The gel was stained with Coommassie blue. MW X 1000 on right.

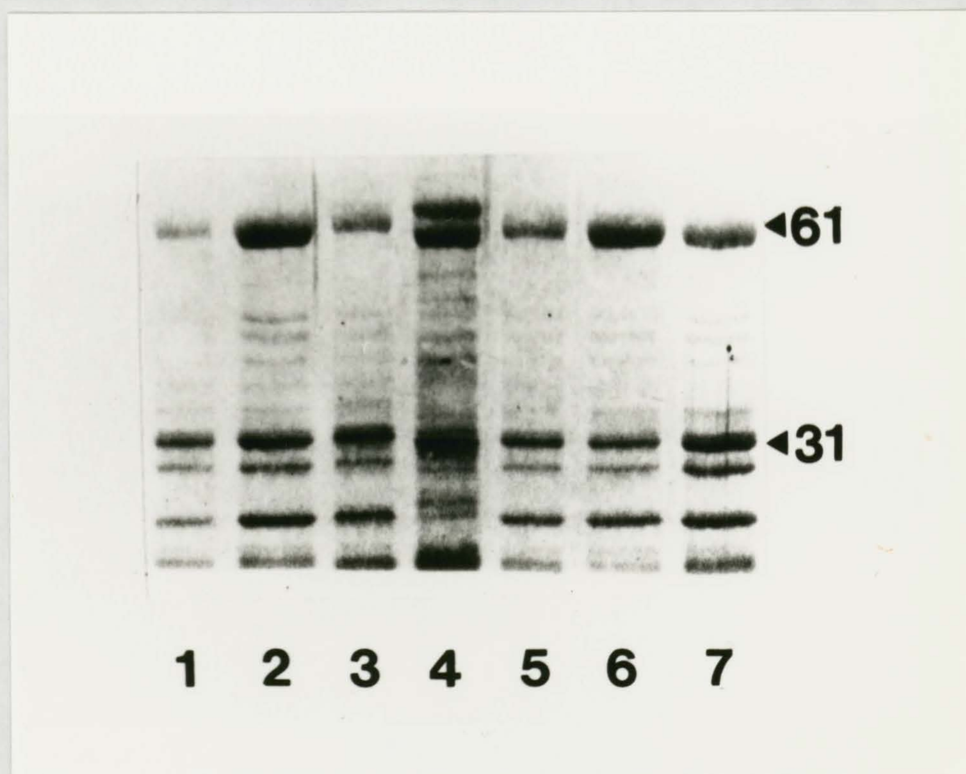
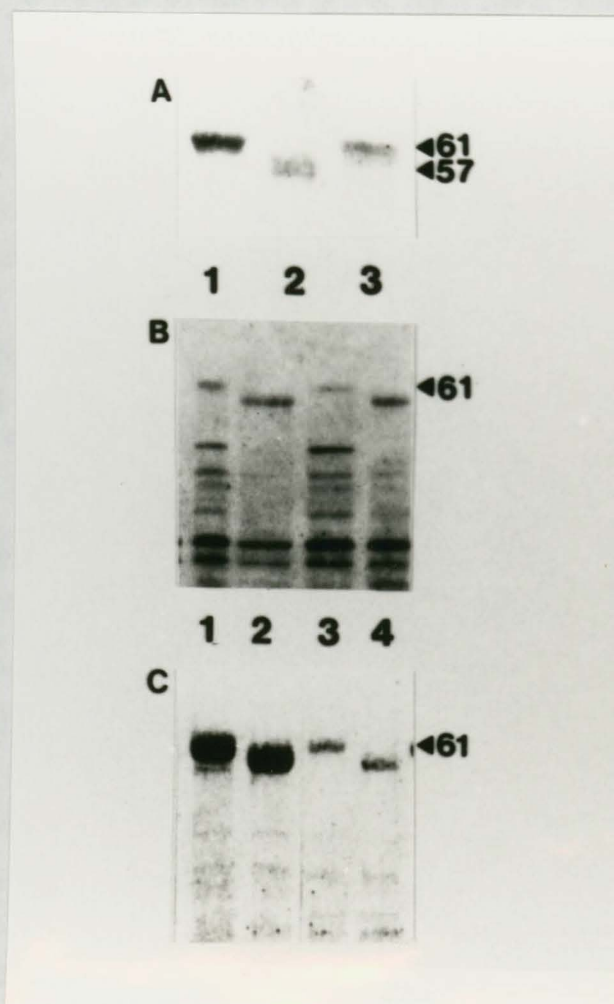


Figure 8. SDS-PAGE (7.5%) of 5 µl of glycine extractable proteins of switched Campylobacter strains. Gel A: lane 1, VC 167 P1; lane 2, VC 167 P2; lane 3, VC 167 P3, obtained by bridge transfer with LAH 2 antiserum. Gel B and C: separation of cells producing phase 2 flagellin by LAH 1 antiserum (B) and LAH 3 antiserum (C); lane 1, VC 159 P1; lane 2, VC 159 P2; lane 3, VC 167 P1; lane 4, VC 167 P2.



the higher MW flagellin was still being produced.

Preparation of antisera to phase 1 flagellin. To confirm the results obtained with Lio 8 antiserum, two additional antisera were prepared to the flagella of VC 167 P1. Two strategies were employed in the production of these antisera. In one case purified phase 1 flagellin was used as the immunogen (antiserum LAH 3). In the other case, live motile cells of VC 167 P1 were used to immunize rabbits (antiserum LAH 1). To increase the specificity of these antisera they were absorbed against homologous heat stable antigens and live VC 167 P2 cells.

Separation of cells producing phase 2 flagellin with LAH 1 and LAH 3 antisera. To confirm the flagellin switch observed with Lio 8 antiserum, LAH 1 and LAH 3 antisera were used to select cells which exhibited motility in the presence of these antisera. This was again accomplished by using the bridging technique previously employed. After two bridge transfers with antiserum LAH 1, SDS-PAGE analysis of glycine extracts showed that each strain produced phase 2 flagellin (Fig. 8B). In the case of LAH 3 antiserum seven transfers were required before each strain was producing phase 2 flagellin (Fig 8C).

The stability of this antigenic variation to subculturing was then determined for the VC 167, VC 156 and VC 159 phase 2 flagellin-producing cells. SDS-PAGE analysis of glycine extracts showed that after ten subcultures the production of the higher MW flagellin remained stable.

Confirmation of flagellin identity. To confirm that the altered MW proteins being examined were indeed flagellin, glycine extracts of cells producing phase 1 or phase 2 flagellin as well as cells switched back

from phase 2 to phase 1 flagellin production were immunoblotted with the *Campylobacter* flagellin MAb 39. This MAb recognizes conserved internal *Campylobacter* flagellin epitopes, and reacted with both phase 1 and 2 flagellins of the Lio 8 type strain VC 167 (Fig 9A) and with both flagellin phases of strains VC 156 and VC 159 (Fig 9B). Polyclonal antiserum SML 2 directed against conserved internal *Campylobacter* flagellin epitopes also reacted with both flagellin phases of all three Lio 8 strains (Fig 9C and 9D).

Immunoprecipitation of flagella phases

Antiserum Lio 8 . To determine if the flagellins of different MW were antigenically different, radio-immunoprecipitation was employed. When radiolabelled glycine extracts of VC 167 P1, VC 167 P2 and VC 167 P3 were immunoprecipitated with Lio 8 antiserum flagellin from VC 167 P1 and VC 167 P3 was immunoprecipitated (Fig 10A, lanes 1,3). No precipitation was observed with phase 2 flagellin (Fig 10A, lane 2). From these results it was evident that the flagellin of VC 167 P3 cells was antigenically similar to the flagellin of VC 167 P1 cells and that both of these flagellins were antigenically different from phase 2 flagellin.

Immunoprecipitations were also performed on glycine extracts of VC 156 and VC 159 cells producing phase 1 and 2 flagella. These results in Fig. 10A (lanes 5,6,7,8) show that predictably the phase 1 flagellins of these strains were immunoprecipitated by the Lio 8 antiserum while phase 2 flagellin was not. These results indicated that the two flagellin phases were serologically distinct, and only flagellin phase 1, carried

Figure 9. Autoradiography of SDS-PAGE immunoblot analysis of flagellin phases of Lio 8 serotype strains with 1:500 dilutions of MAb 39 (Blot A and B) and polyclonal anti-flagellin antiserum SML 2 (Blot C and D). Blots A and C: lane 1, VC 167 P1; lane 2 VC 167 P2; lane 3, VC 167 P3. Blots B and D: lane 1, VC 156 P1; lane 2, VC 156 P2; lane 3, VC 159 P1; lane 4, VC 159 P2. Autoradiogram exposure 24 h. MW X 1000 on right.

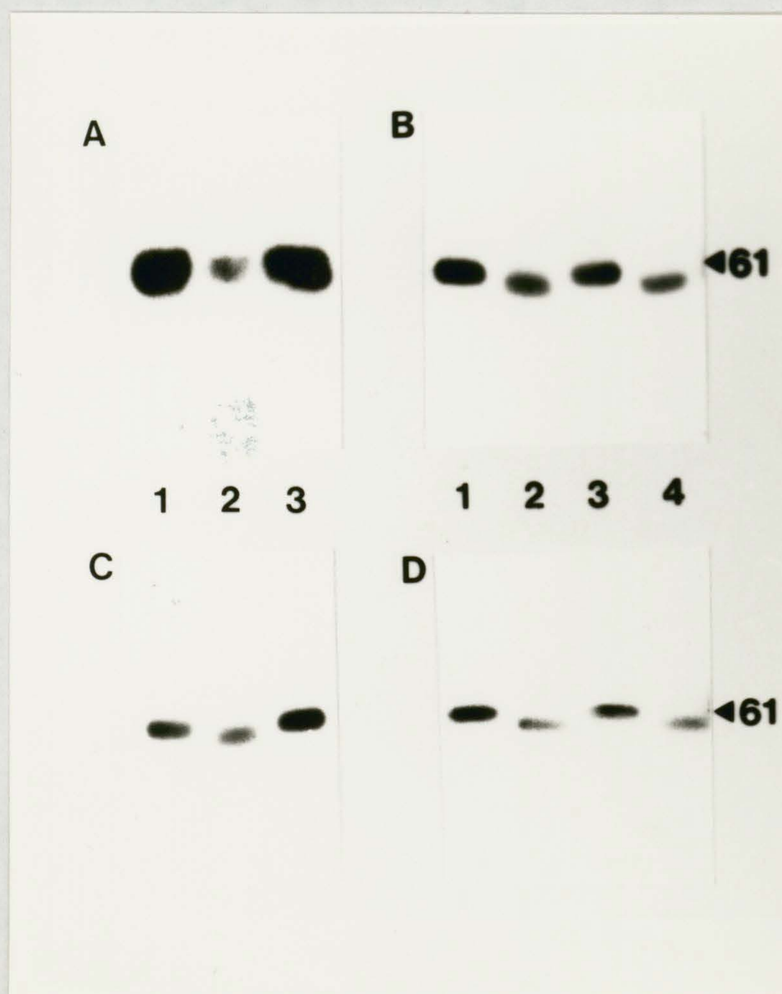
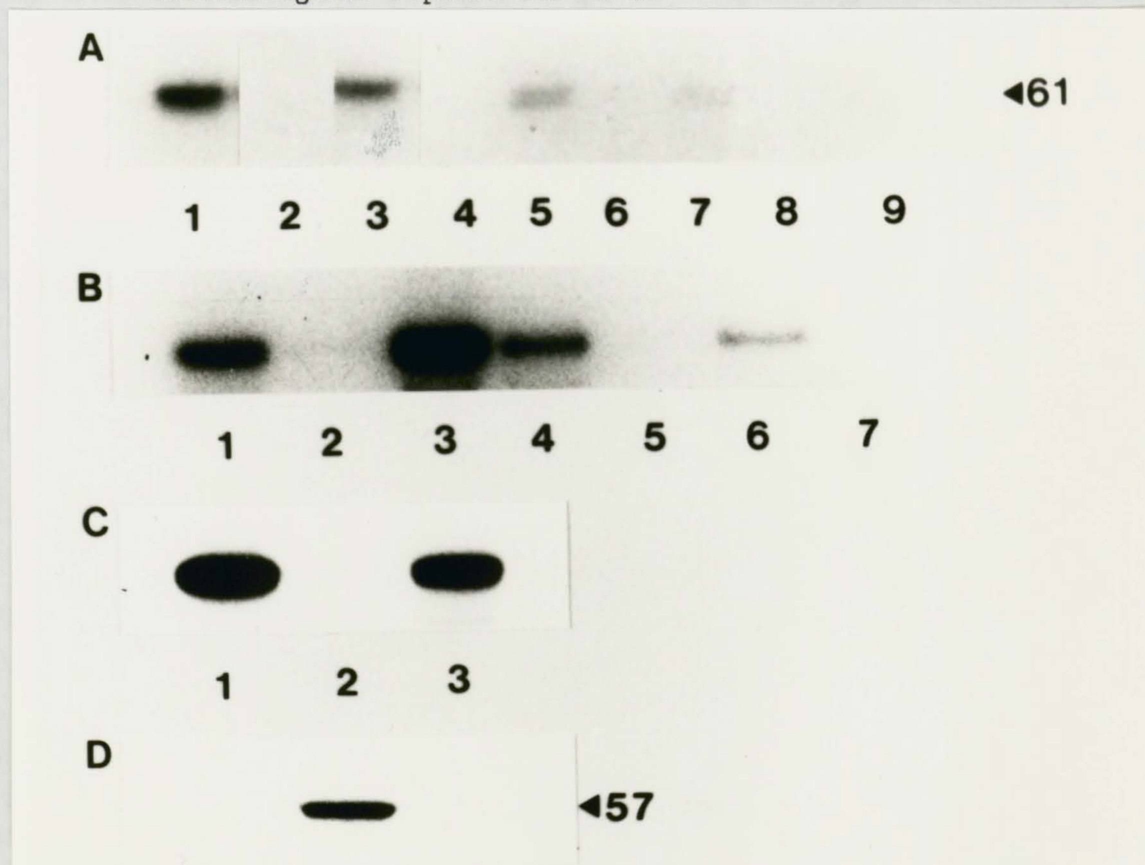


Figure 10. Autoradiogram of SDS-PAGE profiles of glycine-extractable proteins of Campylobacter strains producing different flagellin phases, RIPed with a 1:100 dilution of antiserum. (A) Lio 8, (B) LAH 3, (C) LAH 1, and (D) LAH 2. Autoradiogram A: lane 1, VC 167 P1; lane 2, VC 167 P2; lane 3, VC 167 P3; lane 4 VC 167 P1 RIPed with Lio 29, lane 5, VC 156 P1; lane 6, VC 156 P2; lane 7, VC 159 P1; lane 8, VC 159 P2; lane 9, VC 74. Autoradiogram B: lane 1, VC 167 P1; lane 2, VC 167 P2; lane 3, VC 167 P3; lane 4, VC 156 P1; lane 5, VC 156 P2; lane 6, VC 159 P1; lane 7, VC 159 P2. Autoradiogram C and D: lane 1, VC 167 P1; lane 2, VC 167 P2; lane 3, VC 167 P3. Autoradiograms exposed for 48 h.



epitopes recognized by Lio 8. Controls with an unrelated antiserum and an unrelated flagellin are also shown in Fig. 10A.

Antiserum LAH 3: To extend the results obtained with Lio 8 antiserum, additional antisera were used in RIP assays. The first antiserum used was LAH 3 which had been produced using phase 1 flagellin and was used to immunoprecipitate glycine extractable proteins from VC 167 P1, P2 and P3 cells. This antiserum immunoprecipitated flagellin from glycine extracts of VC 167 P1 and VC 167 P3 cells (Fig. 10B, lane 1,3). Flagellin was not precipitated from VC 167 P2 (Fig 10B, lane 2). These results confirmed the results obtained with Lio 8 antiserum. Also shown in Fig. 10B are immunoprecipitations of glycine extracts from VC 156 and 159 cells producing phase 1 and 2 flagellins. Again only the phase 1 flagellins were immunoprecipitated (Fig. 10B, lanes 4,6). This ability of LAH 3 to immunoprecipitate the phase 1 flagellin from three different Lio 8 strains provided further evidence of the antigenic similarity of the flagellins, although the binding to the homologous flagellin was clearly stronger.

Antiserum LAH 1: RIP assays with LAH 1 antiserum also confirmed the results obtained with antiserum Lio 8 and LAH 3. This antiserum was produced to VC 167 cells with phase 1 flagellin and immunoprecipitated phase 1 flagellin from VC 167 P1 and VC 167 P3 (Fig. 10C, lanes 1,3) but did not immunoprecipitate phase 2 flagellin (Fig. 10C, lane 2).

Antiserum LAH 2: The ability of antiserum LAH 2 to immunoprecipitate flagellin from glycine extracts of VC 167 P1, VC 167 P2 and VC 167 P3 cells was then tested. This antiserum was produced to cells with

phase 2 flagella. The autoradiogram in Fig. 10D shows that this phase 2 antiserum immunoprecipitated phase 2 flagellin but did not precipitate phase 1 flagellin.

Agglutinations of cells producing phase 1 and 2 flagella. To determine the effect of this flagellin antigenic variation on the Lior serotyping scheme, cell agglutinations with Lio 8 antiserum were completed according to the method of Lior (43). The results in Table 5 show that at the recommended antiserum dilution of 1:16, both phase 1 and 2 cells agglutinated. At a 1:32 dilution the antiserum no longer agglutinated phase 2 cells. Therefore strains producing either antigenic phase of flagellin remained Lio serotype 8. This was confirmed by Lior (personal communication). Lior also demonstrated that 167 P1, 167 P2, and 167 P3 were indistinguishable in his biotyping scheme, with all three belonging to *C. coli* biotype 1. These results indicated that at the 1:16 dilution used by Lior for serotyping, epitopes other than those on the flagella were determining the Lio 8 serotype. Interestingly when agglutinations were performed at higher dilutions of antibody, flagella epitopes became more important and could determine serospecificity.

Antisera LAH 1 and LAH 2 were also tested for their ability to agglutinate live *Campylobacter* cells. A 1:16 dilution of antiserum was used in these agglutination tests to illustrate the greater specificity of these antisera compared to the Lio 8 typing antiserum. The results in Table 6 show that antiserum LAH 1 agglutinated cells producing phase 1 flagella, but was unable to agglutinate cells producing phase 2 flagella. In contrast, antiserum LAH 2 agglutinated cells producing phase 2 and did

Table 5. Agglutination of cells producing phase 1 and phase 2 flagella with Lio 8 antiserum.

Dilution of Lio 8	VC167 P1	VC167 P2	VC167 P3	VC156 P1	VC156 P2	VC159 P1	VC159 P2	VC80
1:16	+++[a]	++	+++	++	++	+++	++	-
1:32	++	-	++	++	-	++	-	-
1:64	-	-	-	-	-	-	-	-

[a] +++ indicates good agglutination, ++ is fair agglutination and - is no agglutination

Table 6. Agglutination of cells producing phase 1 and phase 2 flagella with a 1:16 dilution of antisera LAH 1 and LAH 2.

Strain	Antiserum LAH 1	Antiserum LAH 2
VC 167 P1	+++[a]	-
VC 167 P2	-	+++
VC 167 P3	+++	-
VC 156 P1	++	-
VC 156 P2	-	++
VC 159 P1	++	-
VC 159 P2	-	++

[a] +++ is good agglutination, ++ is fair agglutination, and - is no agglutination

not agglutinate cells producing phase 1 flagella.

ELISA analysis of phase 1 and 2 flagellins. To quantitate the antigenic similarities and differences between the phase 1 and phase 2 flagellins, ELISA's were completed using the polyclonal antiserum and monoclonal antibody previously used. The first antiserum employed was polyclonal anti-*Campylobacter* flagellin antiserum SML 2. The results obtained with VC 167 phase 1 and 2 flagellins are shown in Fig. 11A. Both phase 1 and phase 2 flagellins showed similar curves, indicating that both proteins were very similar with respect to their content of epitopes recognized by antiserum SML 2. This similarity was further revealed when anti-*Campylobacter* flagellin MAb 39 was used as both flagellin phases again gave virtually identical ELISA curves (Fig 11B).

When Lio 8 antiserum was employed, the results in Fig. 12A showed that phase 1 flagellin reacted strongly, while phase 2 flagellin gave only a weak reaction. This confirmed that phase 1 flagellin of VC 167 contained epitopes not present on the VC 167 phase 2 flagellin, and these epitopes were recognized by Lio 8 antiserum. To demonstrate that these epitopes were exposed on the surface of the native flagellum filament, Lio 8 antiserum was absorbed with live VC 167 P1 cells. The results in Fig. 12A show that this absorption resulted in a sizable reduction in the ability of antiserum in Lio 8 to react with phase 1 flagellin indicating that the Lio 8 reactive epitopes were surface-exposed. When antiserum Lio 8 was absorbed with live cells producing phase 2 flagellin the antiserum produced curves (Fig. 12B) with only a slight decrease in reactivity, likely due to non-specific absorption. A

Figure 11. ELISA assays of VC 167 phase 1 (●) and phase 2 (○) flagellins (1 μg) reacted with SML 2 polyclonal antiserum (A) and MAb 39 (B). Controls are not shown as their reactivity was below 0.1 absorbance units.

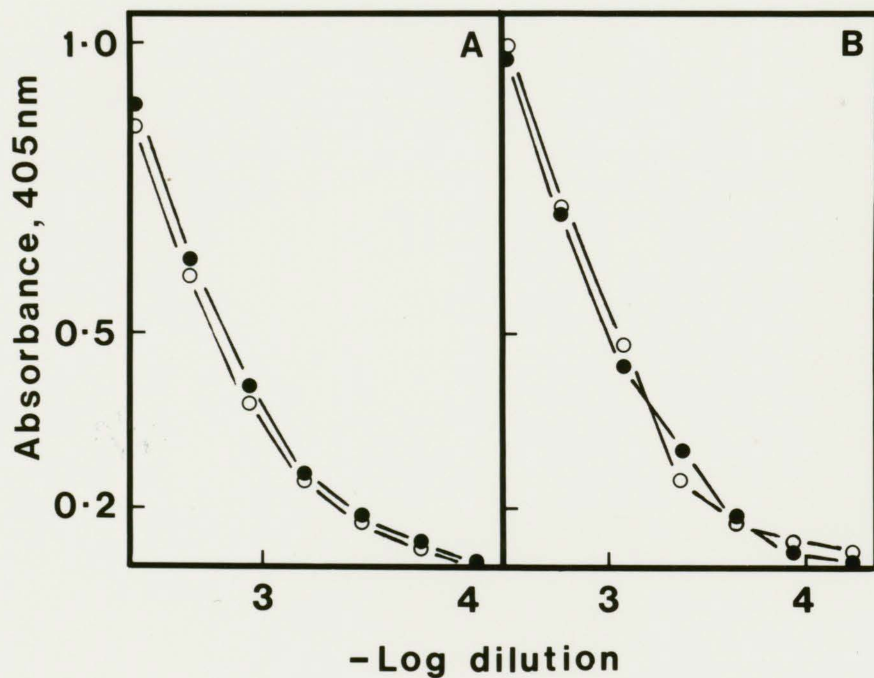
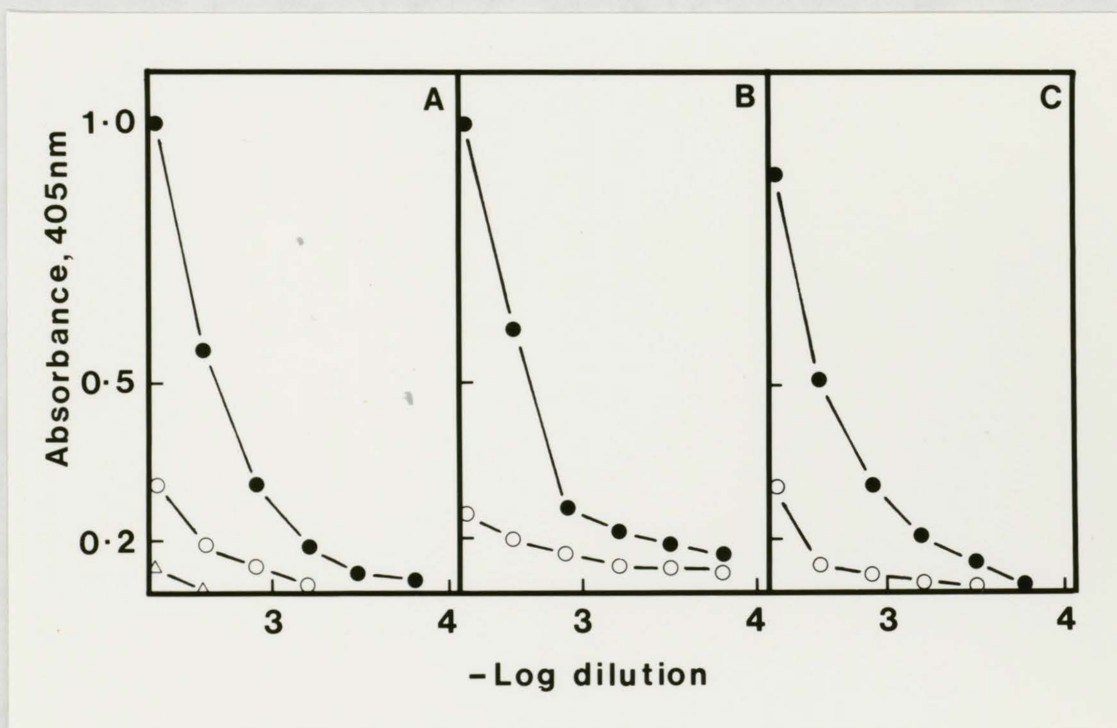


Figure 12. ELISA assays of VC 167 phase 1 (●) and phase 2 (○) flagellins (2 μg) reacted with Lio 8 antiserum (A), phase 1 (△) flagellin reacted with Lio 8 antiserum absorbed with live VC 167 P1 cells, (A), Lio 8 antiserum absorbed with live VC 167 P2 cells (B), and Lio 8 antiserum absorbed with live VC 74 cells (C).



similar small decrease in Lio 8 reactivity was shown after a non-serotype 8 strain was used in a control absorption assay. Despite the slight decrease in reactivity seen in both of these absorptions, the general shape of the curves remained the same indicating that antibodies to major epitopes were not being removed by the absorption (Fig. 12C).

ELISA and absorption experiments were also completed with antisera LAH 1 and LAH 2. Antiserum LAH 1 reacted strongly with phase 1 flagellin and poorly with phase 2 flagellin (Fig. 13A). Only minimal differences in reactivity were observed after absorption with live whole cells of VC 167 P2 (Fig. 13C) or with VC 74 (Fig. 13D). However, when absorbed with live VC 167 cells producing phase 1 flagellin, a dramatic decrease in reactivity was observed (Fig. 13B) confirming that the phase 1 epitopes were surface-exposed on native flagella filaments.

In the case of antiserum LAH 2 only phase 2 flagellin was observed to react, as shown in Fig. 14A. When this antiserum was absorbed with live cells of VC 74 or cells of VC 167 phase 1 very little reduction of titre was observed (Fig. 14B and 14C). However when absorbed with cells producing VC 167 phase 2 flagellin, a sizable reduction in antibodies capable of reaction with phase 2 flagellin was obtained (Fig. 14A). This indicated that antiserum LAH 2 was directed against surface-exposed epitopes on phase 2 flagella.

Frequency of antigenic variation. The frequency of switching of the flagellin antigenic phases, from phase 1 to phase 2 and back, was then determined using thioglycolate motility medium and antisera directed against phase 1 or 2 flagellins. When determining these frequencies,

Figure 13. ELISA assays of 1.0 μg phase 1 (●) and 0.5 μg phase 2 (○) flagellins reacted with LAH 1 antiserum (A), LAH 1 antiserum absorbed with live VC 167 P1 cells (B), LAH 1 antiserum absorbed with live VC 167 P2 cells (C), and LAH 1 antiserum absorbed with live VC 74 cells (D).

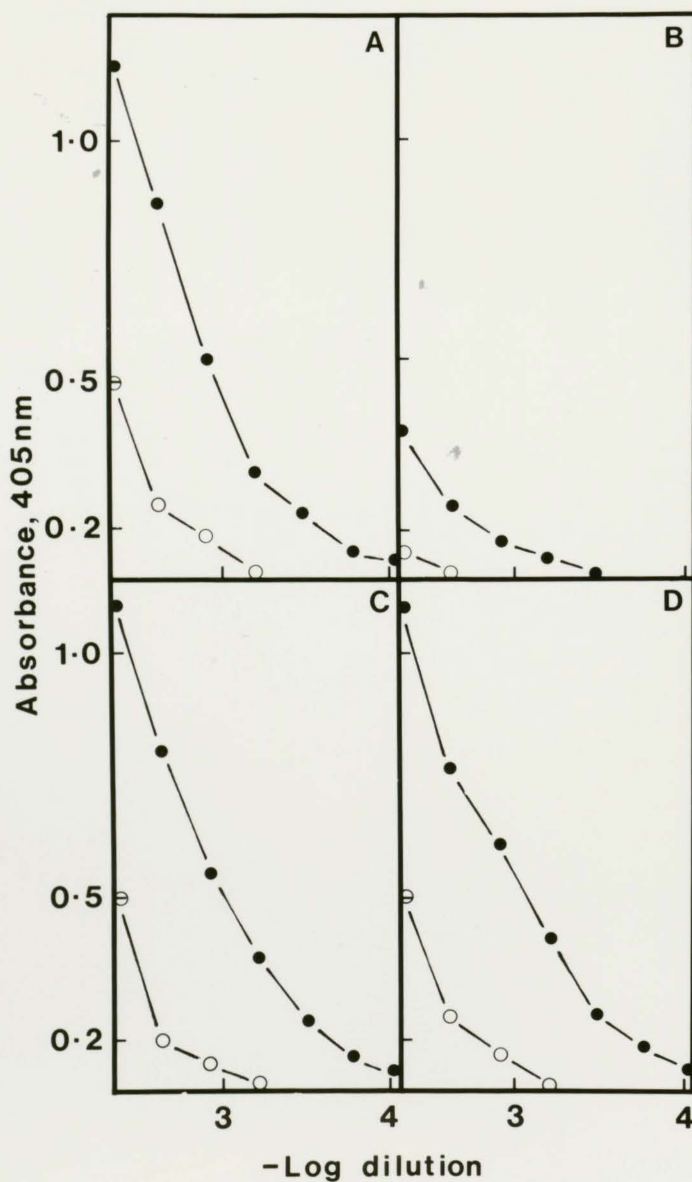
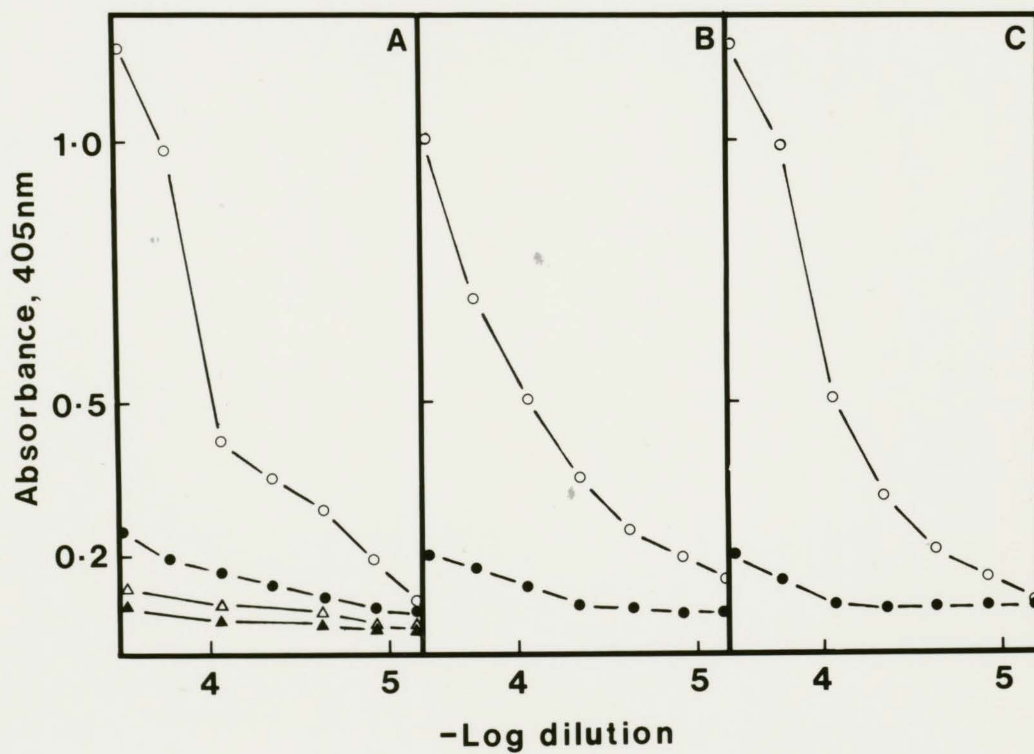


Figure 14. ELISA assays of VC 167 phase 1 (●) and phase 2 (○) flagellins (1 µg) reacted with LAH 2 antiserum (A), LAH 2 antiserum absorbed with live VC 167 P2 cells (A), verus phase 1 (△) and phase 2 (▲) flagellins, LAH 2 antiserum absorbed with live VC 167 P1 cells (B), and LAH 2 antiserum absorbed with live VC 74 cells (C).



the frequency of production of non-motile variants was also determined. The MW of the flagellin produced by all colonies whose motility was not inhibited by the presence of antiserum in the medium, as well as that of 50 non-motile colonies chosen at random was determined by SDS-PAGE following glycine extraction of the Campylobacter cells. Fig. 15 shows one of these gels from plates containing antiserum directed against phase 1 flagellin. In this case the non-motile colonies produced flagellin of MW 61,000. With C. coli VC 167 the frequency of the phase 1 to phase 2 switch was approximately 1.9 x 10^{-5} per cell per generation, while the phase 2 to phase 1 switch was approximately 1.1 x 10^{-6} per cell per generation (Table 7).

Flagellin structural analyses

As the two flagellins were shown to be antigenically distinguishable, structural analysis was performed to determine the extent of structural relatedness of the two proteins.

HPLC purification of flagellin phases. HPLC was used to purify phase 1 and phase 2 flagellins from flagellin preparations of the Lio 8 type strain VC 167 for amino acid composition analysis and in the case of phase 1 flagellin, for N-terminal amino acid analysis. The HPLC purification profiles of the two flagellins from the C8 reverse phase Spheri-10 column are shown in Fig. 16A and B. Both flagellin proteins were eluted at approximately 48% acetonitrile in a TFA/acetonitrile system. As some difficulties were experienced in obtaining pure phase 2 flagellin with the C8 column as assessed by N-terminal amino acid sequence analysis it was also purified using a mono-Q column. Using

Figure 15. SDS-PAGE (7.5%) of 5 µl of glycine extractable proteins from motile and non-motile colonies of the Campylobacter strain VC 167 P1 selected from motility medium in the presence of LAH 1 antiserum (1:500 dilution): lanes 1, 2, 3, 4, and 6, non-motile colonies; lane 5, motile colony.

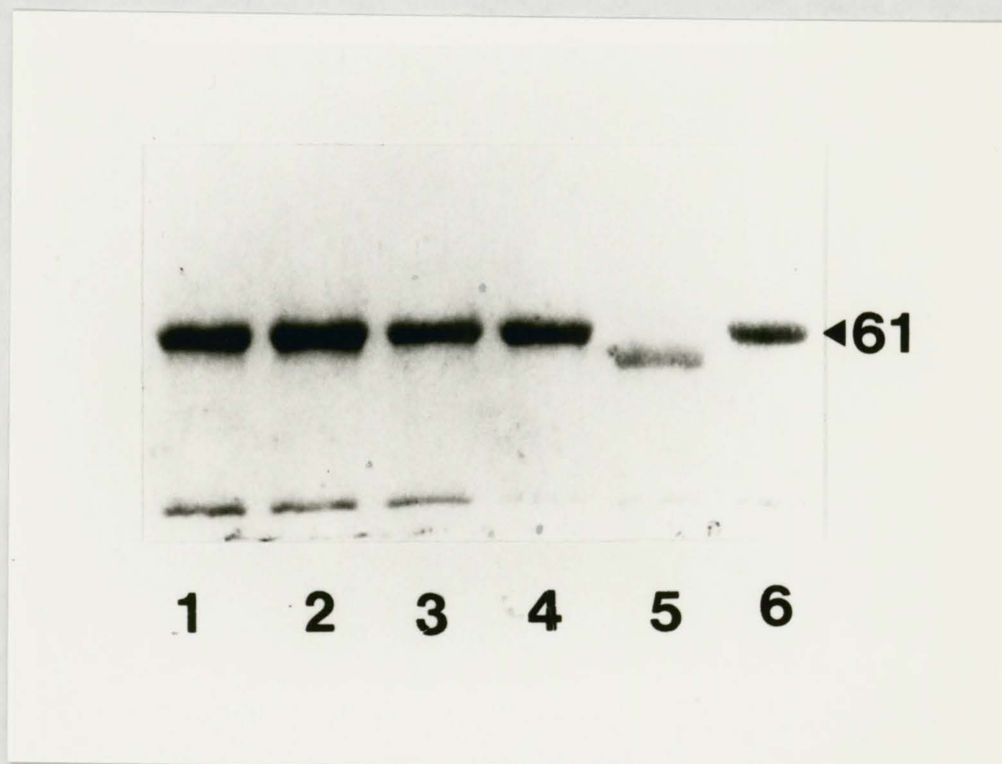
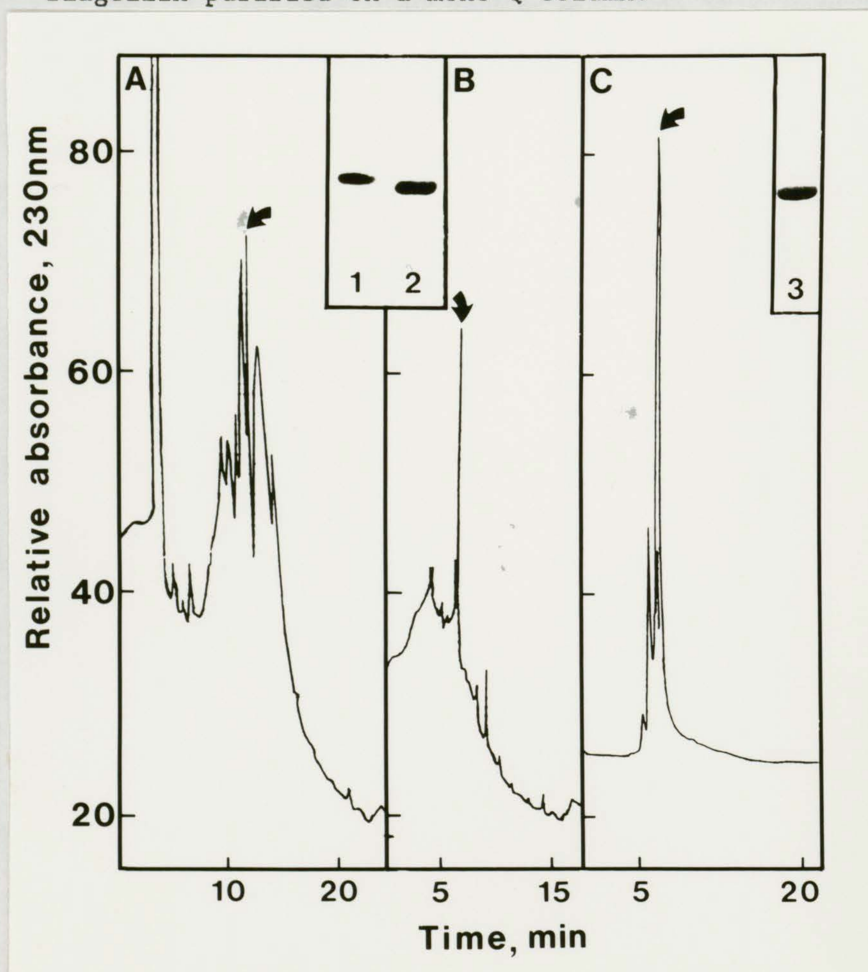


Table 7. Frequency of flagellin antigenic variation in *C. coli* VC 167 P1 and VC 167 P2.

Strain	Variation	No. of variants	Total no. cells examined	Ratio of non-motile cells	No. of generations	Frequency[a]
VC167 P1	P1-P2	19	30,944	1.3×10^{-5}	32	1.9×10^{-5}
VC167 P2	P2-P1	3	64,210	1.1×10^{-5}	31	1.1×10^{-6}

[a] A colony of a given phenotype from a CBA plate was suspended in BHI broth, diluted and plated in motility pour plates to give approximately 200 colonies per plate. The ratio of the number of variants to the total number of cells plated gave the fraction of cells which had undergone transition. The ratio was subtracted by the ratio of non-motile cells produced by the variant. This ratio was then divided by the approximate number of generations the population had undergone to yield the transition rate.

Figure 16. HPLC elution profiles for VC 167 phase 1 flagellin (A) and VC 167 phase 2 flagellin (B) employing a C8 reverse-phase Spheri-10 column and a 0-60% TFA/acetonitrile gradient. An elution profile for VC 167 phase 2 flagellin (C) is also shown employing a mono-Q column and a 0-10% NaCl gradient. The flagellin peak is indicated by an arrow and the inserts on the profiles show SDS-PAGE Coomassie stainings of the purified flagellins. Lane 1, VC 167 P1 flagellin, C8 column; lane 2, VC 167 P2 flagellin, C8 column; lane 3, VC 167 P2 flagellin purified on a mono-Q column.



this ion-exchange column, the flagellin was eluted at 0.1 M NaCl. The HPLC profile of this separation is shown in Figure 16C. The purity of both C8 and mono-Q purified flagellins was confirmed by Coomassie blue staining of SDS-PAGE. In all cases single bands were seen (Fig. 16).

Amino acid composition of phase 1 and 2 flagellins. To determine the structural relatedness of the two proteins amino acid composition analysis was completed. The amino acid compositions of the two purified flagellin molecules are shown in Table 8. Serine, aspartic acid or asparagine, alanine and glycine were found to be in the largest quantities for both proteins. These were followed by glutamic acid or glutamine, threonine, leucine, isoleucine, lysine, valine, and phenylalanine. Arginine, methionine, tyrosine, and histidine were smallest in quantity. No cysteine, proline, or tryptophan were found in either molecule. Although the two molecules had similar compositions, differences did exist. The most noticable differences were with serine, alanine and glycine content. Phase 1 flagellin contained more serine and alanine, while phase 2 flagellin contained more glycine.

N-terminal sequence analysis. N-terminal analysis was then performed on the two flagellins. The results are shown in Table 9, along with the N-terminal sequences of other bacterial flagellins. Although the first two amino acids could not be determined for phase 2 flagellin due to technical difficulties, the next 20 residues of the sequence were identical to that of phase 1 flagellin. Difficulties in analyzing the sequence became apparent after residue 22 for both proteins.

Proteolytic digests of phase 1 and 2 flagellins. To further analyse

Table 8. Amino acid composition of VC 167 phase 1 and phase 2 flagellins.

Amino acid	Residues per flagellin subunit	
	VC 167 Phase 1	VC 167 Phase 2
Asx	80	67
Thr	44	32
Ser	85	63
Glx	56	58
Pro	0	0
Gly	76	109
Ala	79	58
Val	21	25
Met	14	16
Ile	33	35
Leu	34	35
Tyr	10	9
Phe	21	15
His	4	2
Lys	28	31
Arg	17	16
Cys	0	0
Trp	0	0
Total residues per mole	602	571
MW apparent (x 1000)	61.04	57.12
Hydrophobic residues (%) (V,M,I,L,A, and F) (81)	34	32

Table 9. Amino acid sequence of the N-terminal region of flagellin from C. coli strains VC 167 P1 and VC 167 P2, and other bacteria. X represents an undetermined amino acid, - represents shared residues with the C. coli flagellin, . represents a gap inserted to allow sequence alignment.

Organism	Residue
C. coli Phase 1	[1]GFRINTNVAA LNAKANSDLN HRALXQILXV
C. coli Phase 2	[1]XXRINTNVAA LNAKANSDLN XRX
Salmonella (44)	[1]AQV----SLS -LTQN-..-- KSQSALGTA
Bacillus (23)	[1]MX---H-I-- --TLNT..-- RLSSNNSASQK
Caulobacter (32)	[1]ALSV---QP- -I-LQ-..-- RTNDDMQAVQ

the structural similarities and differences between the two flagellin proteins, proteolytic enzyme digests were analysed by SDS-PAGE. The SDS-PAGE gels of these enzymatic digestions are shown in Fig. 17. Trypsin generated several different peptides in the two flagellins as can be seen in Fig. 17 A and B. Partially digested protein is observed in Fig. 17B lanes 1 and 2 (shown as open arrows). One peptide of MW 23,000 was generated in phase 1 which was not observed in phase 2. An HPLC trace of the two digests revealed that four major peptides were generated in phase 2 that were not observed in phase 1. Only one peptide in phase 1 was found to be different than in the phase 2 digest. Chymotrypsin, although producing similar fragmentation patterns did produce a peptide of MW 27,000 in phase 1 which was not observed in phase 2 (Fig. 17C). Proteinase 6 generated identical peptides with both flagellins as observed by SDS-PAGE (Fig. 17D).

Cyanogen bromide was also used to cleave the two flagellin phases of VC 167. One small peptide was generated in phase 1 and not in phase 2. The results of this cleavage can be seen in Fig. 18, which shows the peptide gel of the chemical digestions. Thus differences between the two proteins were also detected using cyanogen bromide, indicating that these two proteins were structurally different from each other in some aspects.

Analysis of LPS of cells producing phase 1 and 2 flagella. Several differences besides flagellin production were also observed in phase 1 and phase 2 producing cells. When plating out cells producing phase 1 and 2 flagella for single colonies, it was observed that phase 1 cells

Figure 17. HPLC (A) and SDS-PAGE (12.5%)(B) analysis of VC 167 phase 1 (1) and 2 (2) flagellins, digested with trypsin. Arrows show differences between the phases. HPLC analysis employed a C8 column with a TFA/acetonitrile system, 0-60% in 60 min. Digests of the two flagellins with chymotrypsin (C) and proteinase 6 (D) were also analyzed by 12.5% SDS-PAGE and stained with Coomassie blue. Lane 1, VC 167 P1; lane 2, VC 167 P2. MW X 1000 on right, for gels B and C the MW markers from the top are at 66, 45, 31 and 21. For gel D, the MW markers from the top of the gel are at 68, 43, 26, 18 and 12.

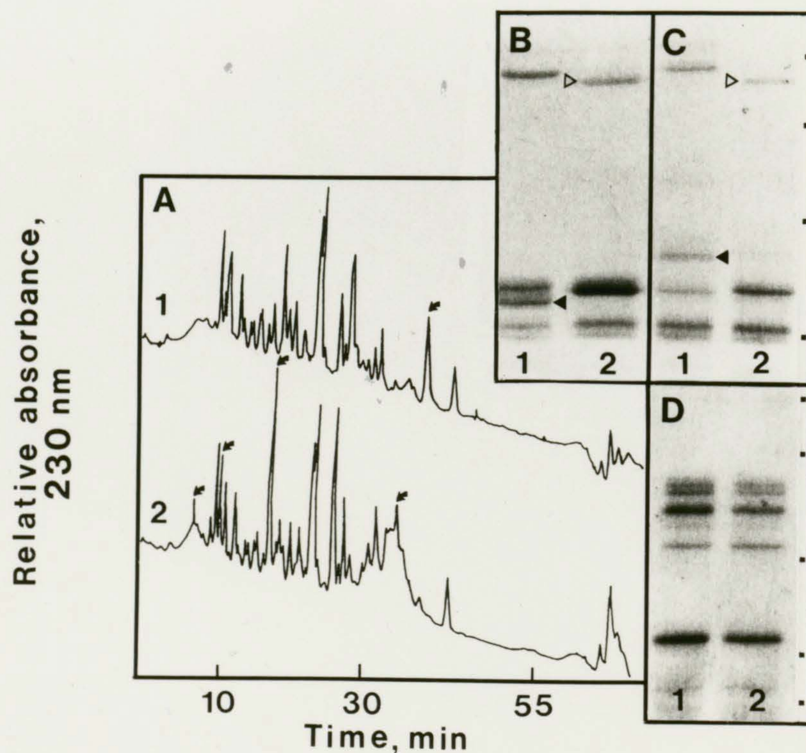
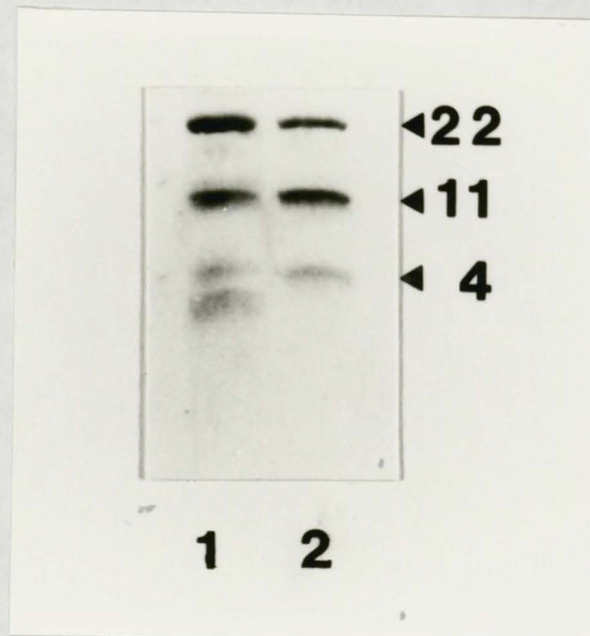
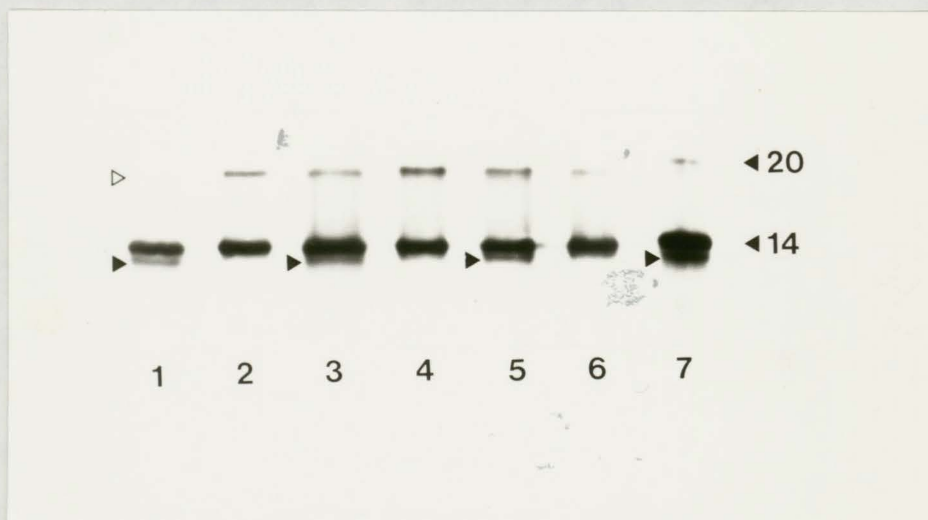


Figure 18. SDS-urea peptide gel analysis of VC 167 phase 1 (lane 1) and phase 2 (lane 2) flagellins digested with cyanogen bromide and stained by Coomassie blue. MW X 1000 on right.



generally appeared to be more opaque and sticky. This suggested that some differences in LPS structure might also be co-occuring with the flagella variation. The LPS profiles of phase 1 and phase 2 producing cells of strains VC 156, VC 159, and VC 167 were therefore examined by SDS-PAGE of proteinase K digested cells. The results are shown in Fig. 19. On examining these profiles it was apparent that in all phase 1 producing cells an LPS molecule (solid arrow) of approximately 9,000 (based on protein standards) was seen that was not found in phase 2 profiles (Fig 19A, lanes 1 to 6). This molecule was present in the profile of VC 167 switched back to phase 1 from phase 2 producing cells (Fig 19A, lane 7). Thus it appeared that variation of flagellin phases was also accompanied by a change in the LPS structure of all three strains tested. The higher MW LPS molecules seen in Fig. 19 (hollow arrows) are believed to be aggregates of the lower MW LPS observed at 11,000 (based on protein standards) in all strains (66).

Figure 19. SDS-PAGE (12.5%) profiles of Campylobacter strains digested with proteinase K and silver stained for LPS visualization. The MWs shown are based on protein standards. The solid arrows are pointing to unique phase 1 LPS and the hollow arrow is showing the location of LPS aggregates. Lane 1, VC 156 P1; lane 2, VC 156 P2; lane 3, VC 159 P1; lane 4, VC 159 P2; lane 5, VC 167 P1; lane 6, VC 167 P2; lane 7, VC 167 P3. MW X 1000 on right.



DISCUSSION

Of the many serological typing schemes which have been proposed for identification of thermophilic Campylobacters, the serotyping scheme developed by Lior has gained perhaps the widest acceptance, at least amongst the systems based on thermolabile antigens. One factor in the wide acceptance of the Lior system is the fact that it uses slide agglutinations of live bacteria and so is easy to perform. Little is known concerning the identity of the molecules on the surface of the *Campylobacter* cell which contribute to serospecificity in the Lior scheme. This study examined the contribution of flagella to the serospecificity of one of the most commonly isolated human serotypes, Lio serotype 8.

This study revealed that while the Lio 8 serotyping antiserum contained high titers of antibodies which recognized the flagella of some Lio 8 strains, the serodeterminants for serotype Lio 8 were not flagella-borne. Although the molecules responsible for Lio 8 serospecificity were not identified, IFAT assays at the 1:16 antiserum dilutions employed by the Lior scheme showed considerable cell surface fluorescence suggesting that outer membrane or other surface associated proteins were responsible. The inability of the Lio 8 antiserum to react with these components in immunoblot analyses indicated that the serodeterminant epitopes were conformation-dependent. Similarly the serodeterminants of the related serogroup Lio 29 appeared to be conformational and again were localized on the cell surface and not on the flagellum.

Flagella epitopes recognized by the Lio 8 antiserum were also conformational, and surface-exposed, and RIP analysis localized these epitopes on a flagellin of subunit MW 61,000. However immuno-electron microscopic evidence together with 7.5% SDS-PAGE analysis of glycine extracted flagellin of Lio 8 strains showed that many strains had the ability to produce an antigenically distinct flagellin molecule, and in addition to producing the MW 61,000 flagellin, were able to produce MW 57,000 flagellin. This MW 57,000 flagellin was not immunoprecipitated by the Lio 8 antiserum.

This suggested that the Lio 8 Campylobacter cultures contained some cells producing flagella composed of flagellin monomers of one MW and others producing flagella composed of flagellin monomers of different MW, and that flagellin production was subjected to phase variation. This was confirmed when antibody-mediated immobilization allowed the selection of cells producing flagella in a different antigenic phase. The type strain VC 167 produced MW 61,000 flagellin before antibody selection but after antibody selection produced flagellin of MW 57,000. This was repeated several times and was also demonstrated with two other Lio 8 serogroup strains. Although this 57,000 MW flagellin still reacted with polyclonal antiserum to internal conserved Campylobacter flagellin epitopes and to a monoclonal antibody which also recognizes a cross-reactive internal Campylobacter flagellin epitope (Logan and Trust, submitted), it showed altered antigenic specificity as quantitated by ELISA's with Lio 8 antiserum. This altered antigenic specificity was confirmed with two additional antisera (LAH 1 and LAH 3)

which specifically bound to surface epitopes on the phase 1 flagellin as determined by RIP, ELISA, and agglutination reactions.

Antiserum LAH 2, produced against surface epitopes on phase 2 flagella was also able to distinguish between the two flagellin molecules, as shown by its ability to specifically recognize the phase 2 flagellin in RIP experiments. This antiserum also reacted by ELISA with only phase 2 flagellin and agglutinated cells producing only phase 2 flagella. Antiserum LAH 2 allowed the reverse selection to be made. That is VC 167 cells with flagella in phase 1 were selected from cells producing phase 2 flagella.

The availability of antisera specific to the two flagellin phases also allowed for the estimation of the frequency of flagellin switching. In the case of the type strain the frequency of the phase 1 to phase 2 switch was approximately 1.9×10^{-5} per cell per generation (17,26,93,103), while the phase 2 to phase 1 switch frequency was approximately 1.1×10^{-6} per cell per generation. These rates are comparable to those found by Stocker (103) in _Salmonella_. As Stocker observed with _Salmonella_ flagella phase variation, the frequency of transition of the _Campylobacter_ switch in one direction was different than the frequency in the reverse direction.

Structural analysis was then performed on the purified flagellins to determine their relatedness. The amino acid compositions revealed that the two phases were very similar, although differences in amino acid content existed with serine, glycine and alanine. The hydrochloric acid hydrolysis further revealed that the amino acid compositions of the two

Campylobacter flagellins were similar to those of other bacterial flagellins (75,88,92). For example, cysteine and tryptophan are always absent. The hydrophobicities of the various flagellins when determined from the amino acid compositions are very similar, between 32-34%. From these calculations it was concluded that the Campylobacter flagellin molecule is not very hydrophobic (81).

The amino terminal sequences of the two flagellins were found to be highly conserved. Although the first two amino acids were not resolved for phase 2 flagellin, the next 20 amino acids were identical to the phase 1 sequence. Importantly, N-terminal sequence analysis of flagellins from two other strains of C. coli and C. jejuni also showed sequences identical to these two flagellins (Logan and Trust, unpublished). Comparison of the phase 1 and 2 flagellin N-terminal sequences of C. coli VC 167 with those of Salmonella (44,45,106), Bacillus (19,23,24,89), and Caulobacter (32) also revealed considerable sequence homology. As the flagellin's N-terminal is conserved this suggests that important functional roles may exist for this end of the molecule. Interestingly DNA sequence analysis of the flagellin genes of Salmonella have shown conservation of the C-terminal ends of the molecule as well (114).

In addition to the sequence conservation at the N-terminal end of the flagellin molecule, analysis of the peptides generated by proteinase and by cyanogen bromide hydrolysis indicated that other regions of the flagellin molecules were also conserved. This was especially evident with proteinase 6 where SDS-PAGE analysis showed that identical cleavage

patterns of the two flagellins were produced. With both, flagellin peptides of MW 37,000, 36,000, 33,000, 27,000, 17,500 and 14,000 were generated. Digestion with trypsin or chymotrypsin also produced similar HPLC and SDS-PAGE profiles with apparently identical peptides of MW 54,000, 53,000, 24,500, 21,000, and 20,000. Similarily, cyanogen bromide hydrolysis generated peptides of MW 22,000, 11,000, and 4,000 in both flagellins. Cyanogen bromide analysis of other Campylobacter flagellins (Logan and Trust, submitted) have also shown apparently conserved peptides of similar MW to those found with the two flagellins examined here. Immunochemical evidence showed that these conserved polypeptides were not exposed on the surface of the native flagellum filament.

In addition to these structural similarities, cleavage by CNBr, and by both trypsin and chymotrypsin, revealed structural differences between the two flagellins. In the case of cyanogen bromide hydrolysis, a peptide of low MW was shown in phase 1 but not phase 2 flagellin. In the case of trypsin, SDS-PAGE analysis showed unique peptides of MW 23,000 with phase 1 flagellin, while in the case of chymotrypsin a unique peptide was generated at 27,000. While the location of these unique peptides was not determined, it seems likely, since the antigenic differences reside on the surface of the intact flagellin filament, that the unique peptides are surface-exposed. Analogy with the flagellin structure of Salmonella suggests that these peptide differences may be centrally located in the flagellin monomer.

The genetic basis for this flagellin phase variation is not clear at

this time, and will require the molecular cloning of the flagellin genes. Other studies have shown that phase variation is accompanied by a rearrangement of the genomic DNA of VC 167 (Guerry and Trust, unpublished). Results obtained with an oligonucleotide probe to the N-terminal sequence of the Campylobacter flagellin suggest that this rearrangement does not involve this region of the flagellin gene. The DNA rearrangement may involve the insertion of a large plasmid into the VC 167 chromosome during the switch from phase 1 to phase 2, and the excision of this plasmid during the switch from phase 2 to phase 1 (unpublished results). These genomic alterations which appear to accompany the flagellin phase variation likely also account for the other phenotypic changes such as colony morphology, that accompany the flagellin phase variation.

The most important of these other changes with respect to both pathogenesis and serotyping is the apparent phase variation in LPS structure that accompanies the flagellin phase variation and DNA rearrangement. While not the focus of this study, and so not analyzed in depth to date, LPS produced by strains in phase 1 contained a low MW molecule, which was not observed in phase 2 strains. Such LPS alteration has also been observed in Neisseria gonorrhoeae where it appears to be associated with induced resistance of the bacteria to killing by human serum (107). A similar role may exist for the LPS change observed with Campylobacter. Certainly experiments by Perez Perez and Blaser (85) have indicated that LPS composition may be an important determinant of serum susceptibility among Campylobacter species. Antigenic changes

in the LPS would also have important ramifications in the Penner serotyping system. While it is not clear at this time that the LPS and flagellin phase variations are linked, it is clear that both the Lior and Penner serotyping schemes require reexamination and a system which incorporates both antigenic types and accounts for phase variation in both would seem to be required.

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FLAGELLA PHASE VARIATION IN THE THERMOPHILIC CAMPYLOBACTERS

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