BIOCHEMICAL AND IMMUNOLOGICAL CHARACTERIZATION OF THE FLAGELLA PROTEIN OF *CAMPYLOBACTER PYLORI*

by

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We accept this thesis as conforming
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ABSTRACT

In 1987, Lee and coworkers (Infect. Immun. 55:828-831) reported that a *Campylobacter pylori* protein of subunit molecular weight (Mr) 57-59,000 (57-59 kDa) reacted in Western blots with an antiserum (SML2) directed against the flagellin of *Campylobacter jejuni*. Subsequent reports by Newell (J. Gen. Microbiol. 133:163-170) and Mills et. al. (J. Clin. Microbiol. 26: 1411-1413) of a cross reacting antigen among campylobacters supported the suggestion that the protein being recognized by the anti-*C. jejuni* flagellin antiserum was the *C. pylori* flagellin, while reports by Geis et al. (J. Clin. Microbiol. 27:436-441), Perez-Perez and Blaser (Infect. Immun. 55:1256-1955)and Dunn et. al. (Infect. Immun. 57:1825-1833) disputed the findings. This study was initiated to clarify whether or not the 57-59 kDa antigenically cross-reactive protein of *C. pylori* was flagellin, or whether the antigenic cross reactivity was due to determinants shared by an unrelated protein.

To answer this question, flagellar filaments were isolated from *C. pylori* 5294 using two principles of separation: CsCl density gradient ultracentrifugation, and pH 2.0 acid dissociation and differential ultracentrifugation. In both cases a 58 kDa subunit was the predominant protein recovered and in both cases this protein was shown to react by western blotting with antiserum (SML2) directed against the flagellin protein of *C. jejuni*. The 58 kDa subunit protein was purified to homogeneity using a Mono Q anion exchange column, and N-terminal amino acid sequence analysis showed significant identity with the N-terminal amino acid sequence of the 59.5 kDa flagellin of *C. coli* VC167B, and sequence relatedness with the N-terminal sequences of flagellins of *Salmonella* species

and *Bacillus* species. The amino acid composition of the 58 kDa protein subunit of *C. pylori* was also typical of a flagellin molecule. Particularly significant was the absence of cysteine and tryptophan residues and the low content of proline residues which are features characteristic of other flagellins.

Polyclonal antisera and monoclonal antibodies directed against the 58 kDa protein were produced, and indirect immunofluorescence and immunogold electron microscopic studies showed that the antibodies bound to the surface of the inner flagellin filament. Cell absorption studies with polyclonal antisera confirmed that the sheath did not contain the 58 kDa protein. Immunochemical analysis with polyclonal antiserum against *C. pylori* flagellin verified the presence of cross reactive epitopes among *Campylobacter* flagellins. This antigenic cross-reactivity did not however extend to the flagellins of other gram-negative bacteria. Monoclonal antibody (Mab) 72c was also specific for a linear epitope shared by *Campylobacter* flagellins. This epitope was exposed on the surface of the inner flagellin filament of *C. pylori*, but was not surface-exposed on the unsheathed flagella filament of *C. coli* VC167B, indicating that the flagella filaments of the two species were assembled in a different fashion. In addition to this shared epitope, western blotting, ELISA, immunogold electron microscopy, and indirect immunofluorescence studies showed that the 58 kDa flagellin protein of *C. pylori* carried two additional species-specific epitopes. Both epitopes were exposed on the surface of the inner flagellin filament. The epitope for MAb 220a was linear, while the epitope for MAb 104a appeared to be topographically assembled.

Examiners: [redacted]

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LIST OF ABBREVIATIONS

Amino Acids

Glycine	gly	G
Alanine	ala	A
Valine	val	V
Leucine	leu	L
Isoleucine	ile	I
Serine	ser	S
Threonine	thr	T
Aspartic acid	asp	D
Asparagine	asn	N
Lysine	lys	K
Glutamic acid	glu	E
Glutamine	gln	Q
Arginine	arg	R
Histidine	his	H
Phenylalanine	phe	F
Cysteine	cys	C
Tryptophan	trp	W
Tyrosine	tyr	Y
Methionine	met	M
Proline	pro	P

Antisera

Polyclonal

AL1	polyclonal antiserum against formalinized cells of *C. pylori* 5155
JB1	polyclonal antiserum against formalinized cells of *C. pylori* 5294
JB2	polyclonal antiserum against SDS-denatured flagellin of *C. pylori* 5294
JB3	polyclonal antiserum against Mono Q purified flagellin of *C. pylori* 5294
SML2	polyclonal antiserum against SDS-denatured flagellin of *C. jejuni* VC74

Monoclonal	MAbs against Mono Q purified purified flagellin of *C. pylori* 5294
72c	
104a	
220a	

Chemicals

BSA	bovine serum albumin
C	carbon
CAPS	3-cyclohexylamino-1-propanesulfonic acid
CsCl	cesium chloride
CO_2	carbon dioxide
$CuSO_4$	copper sulphate
DNAse	deoxyribonuclease
EDTA	ethylenediaminetetraacetic acid
HAT	hypoxanthine aminopterine thymidine
HCl	hydrochloric acid
HT	hypoxanthine thymidine
$MgCl_2$	magnesium chloride
N_2	nitrogen
NaN_3	sodium azide
NaCl	sodium chloride
NaOH	sodium hydroxide
NH_4OH	ammonium hydroxide
O_2	oxygen

PBS	phosphate buffered saline, pH 7.4
PTA	phosphotungstic acid
RNAse	ribonuclease
SDS	sodium dodecyl sulfate
TBS	TRIS buffered saline, pH 7.4
TRIS	tris(hydroxymethyl)aminomethane

Methods

FPLC	fast protein liquid chromatography
IFAT	indirect immunofluorescence
Mono Q	anion exchange column
pI	isoelectric point
RIA	radioimmune assay
RFP	restriction fragment polymorphism
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

Organisms

CLO	*Campylobacter*-like organisms

Structures

C-	carboxy terminal
LPS	lipopolysaccharide
N-	amino terminal
OM	outer membrane
w/c	whole cell lysate

Units of concentration

M	moles/L
w/v	weight/volume
v/v	volume/volume

Units of Measurement

A	ampere
c	centi-
C	Celcius
g	gram
x g	gravity
hr	hour
kDa	kilodalton
L	litre
µ	micro-
m	milli-
m	meter
min	minute
n	nano-
sec	second

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There are many people at the University of Victoria who have helped me during the past three years. Of course, the most important is my supervisor, Dr. Trevor J. Trust. I cannot thank him enough for giving me this opportunity to study in his laboratory. By a delicate balance of instruction and independence, he has guided me throughout this project. He has taught me how to design and perform experiments, how to analyze data, and how to present data. But more importantly, Dr. Trust has instilled in me an excitement and deeper appreciation for science, the importance of organizing my time, the difference that details can make, and the success that can follow as a result of perseverance. It is these last qualities to which I am most indebted to Dr. Trust

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DEDICATION

To my mom and dad

INTRODUCTION

Culture of biopsy specimens of the human gastric mucosa enabled Warren and Marshall in 1982 to successfully isolate a gram-negative spiral microaerophilic bacteria from 70% of 40 patients with gastric ulcers and from 90% of 70 patients with duodenal ulcers (70). Because the organism grew on media routinely used to culture the enteropathogenic *Campylobacter jejuni,* and because the organism was initially isolated from the pyloric region of the stomach, it was named *Campylobacter pyloridis* (70). Subsequent realization that the name was linguistically incorrect resulted in an alteration of the species name to *pylori.* Partial 16S rRNA sequencing studies then revealed that *C. pylori* did not belong to the genus *Campylobacter* as it was phylogenetically closer to the genus *Wolinella* (68, 110, 120). However, comparison of five major taxonomic features showed that *C. pylori* was markedly different from other bacteria in the genus *Wolinella* (42). As a result, it was recently proposed that *C. pylori* be transferred to a new genus, *Helicobacter* (42). Future useage will decide whether the organism will be called *C. pylori* or *H. pylori.* This thesis will use the name *C. pylori.*

The numerous epidemiological studies which followed Warren and Marshall's initial observation of the association of *C. pylori* with inflammatory gastroduodenal conditions has established, in the opinion of many workers, that the organism is causally associated with active and chronic gastritis, and peptic ulcers, and is likely a contributor to duodenal ulcers as well as gastric carcinoma (58). Indeed two human volunteer studies have provided convincing evidence for the pathogenic role of *C. pylori* (90, 102). Ten days after swallowing an inoculum of *C. pylori* cells,

active chronic gastritis associated with an infiltration of polymorphs was found in endoscopic biopsies taken from both volunteers. Of particular importance was the appearance of a chronic inflammatory condition which is the hallmark of *C. pylori* infections (58).

Biopsy studies have shown that *C. pylori* specifically colonizes the gastric mucosa of humans, and recent estimates indicate that the organism is present in the stomach of more than 56% of the world's population over 40 years of age (45, 70). In many of these individuals, inflammatory gastroduodenal disease of one form or other is apparent. It therefore appears that *C. pylori* is one of the most common bacterial pathogens of humans. The enormous importance of gastric colonization by *C. pylori* is illustrated by the fact that inflammatory gastroduodenal conditions, including gastric ulcer diseases, are among the most common human ailments requiring medical consultation and therapy throughout the world.

There are significant economic and social costs associated with the hospitalizations, serial endoscopies, and blood transfusions resulting from gastrointestinal hemorrhage in *C. pylori* colonized patients. There is clearly a need for effective prophylactics to prevent colonization of the gastroduodenal tract by *C. pylori*. The most desirable prophylactic would obviously be a *C. pylori* vaccine. There is also a need for a variety of diagnostic reagents which will allow rapid identification and differentiation of *C. pylori* at the Genus, species, or strain level, as well as purified antigens which can be used to detect the presence of antibodies to *C. pylori* in patients' serum. The availability of such serological reagents and purified antigens would not only facilitate the identification of infection and the organism at

the clinical laboratory level, but would provide much needed information on the epidemiology of C. *pylori*.

However, the rational development of an effective vaccine, the provision of effective diagnostic serological probes for *C. pylori*, and the provision of purified antigens will require a detailed knowledge of the antigenic structure of the *C. pylori* cell surface. In addition, there is a secondary potential benefit that will result from the availability of purified antigens, and antibodies used for their characterization, namely these probes should facilitate the molecular cloning of *C. pylori* genes. This in turn could provide for nucleic acid diagnostic probes (101) at the Genus, species, or strain level, and certainly should facilitate molecular genetic studies aimed at understanding the molecular basis for the virulence of this important pathogen (17). For example, motility imparted by the flagella has been suggested as a virulence mechanism because it should allow the organism to move through the mucus blanket to its specific attachment sites on the gastric mucosa (73). Cloning of the flagellin gene would greatly facilitate the isolation of non-motile mutants to determine the role of motility in *C. pylori* virulence.

At the start of this study, little was known concerning the antigenic structure of *C. pylori*. Although a number of proteins of different Mr had been shown to be antigenic in immunoblot studies (141), the actual molecular identity of these various proteins were not established. Early studies by Lee and coworkers (72) had shown that only one protein of *C. pylori* was consistently cross-reactive between *C. pylori* and enteropathogenic campylobacters. This protein had a subunit Mr of 57-59 kDa in *C. pylori*, and displayed antigenic cross reactivity in immunoblots with an antiserum

produced against the *C. jejuni* flagellin, the immunodominant protein antigen in *C. jejuni* infections (87). This finding lead to the suggestion that the antigenically cross-reactive 57-59 kDa protein was *C. pylori* flagellin. This was supported by Newell (106) and Mills et. al. (97). However Dunn and coworkers (23), and Geis et. al. (38) suggested that *C. pylori* flagellin might not in fact be the protein which displayed antigenic cross-reactivity with anti-*C. jejuni* flagellin antiserum, or might have a subunit Mr lower than the protein identified by Lee et. al. (72).

The objective of this thesis study was therefore to establish the identity of *C. pylori* flagellin. To do this, flagellar filaments were isolated from a typical gastritis isolate of *C. pylori*, and the flagellin contained in these filaments was purified to homogeneity. The biochemical identity of the purified 58 kDa flagellin was then established by N-terminal sequence analysis and amino acid composition analysis. Polyclonal and monoclonal antibodies were than raised to the purified flagellin to provide immunological and morphological confirmation of flagellin identity, as well as information on the structure of the sheathed flagella, and the antigenic structure of the inner flagellin filament of *C. pylori*. This thesis reports on the findings of the study.

LITERATURE REVIEW

Discovery. The first reported observations of spiral bacteria in the stomach were by Bizzozero in 1893, from the stomachs of animals, and by Salomon in 1896, from the stomachs of humans (71). Since then, there had been occasional observations that confirmed the initial reports, but not much significance was placed on them (64). This was because it was not realized that the presence of spiral organisms might correlate with a diseased state of the stomach. It was not until 1975 when gastroenterologists were able for the first time to view the mucosa of the human stomach, that this correlation became apparent (64, 89). Interest in these spiral organisms was sparked when Steer and Colin-Jones observed that numerous gram-negative bacteria were present in the gastric mucosa of 80% of patients with a gastric ulcer and that the mucosa was characterized by an infiltration of polymorphs signalling active inflammation. The major thrust to research in this area came in 1982 when Warren and Marshall successfully isolated the organism in question from 70% of 40 patients with gastric ulcers and from 90% of 70 patients with duodenal ulcers (89). As is often the case in science, serendipidity played a role in the culturing. Previous attempts at culture were unsuccessful because the plates were incubated only for 48 hours, but because of an Easter holiday, one set of plates was left to grow for five days microaerophilically (70).

The organism. The bacterium that Marshall cultured was gram-negative, spiral shaped, microaerophilic, motile and catalase positive. The organism has since been described as sometimes being U-shaped and appearing as coccoid forms after prolonged culture. The organism is approximately 3.5 μm long by 1 μm wide, with larger forms (3.48 μm) being

isolated from the antrum of the stomach, and smaller forms (2.62 μm) being isolated from the duodenum (70, 59). Because the bacteria grew on media routinely used to culture *Campylobacter jejuni* and because the organism was initially isolated from the pyloric region of the stomach, it was named *Campylobacter pyloridis* (70). Subsequent realization that the name was linguistically incorrect resulted in an alteration of the species name to pylori. Three characteristics define the genus *Campylobacter*: 1. G + C mol % content of DNA 2. spiral shape and 3. requirement for microaerophilic growth atmosphere. Consistent with this definition is the fact that *C. pylori* has a G + C mol % content of 35.8 - 37.1 which is within the accepted range of 33 to 35% for campylobacters (68). The organism also has a spiral shape, requires microaerophilic conditions for growth, and like other campylobacters, *C. pylori* is unable to metabolize sugars by fermentation or by oxidation (70).

As *C. pylori* was studied in greater detail, major differences were revealed that made it apparent that this bacterium was not a true member of the genus *Campylobacter*. Ultrastructural studies showed that *C. pylori* has five to six sheathed flagella that arise from a single pole (11). In addition, the flagellae have bulbs at their ends which are similar to structures seen in vibrios (11, 91). In comparison, other campylobacters are motile by means of a single, unsheathed flagellum, which arises from a distinct pit-like depression from either pole of the cell (44). Ultrastructural studies also revealed that *C.pylori* has a smooth cell wall, which is unlike other *Camplobacter* species which are described as having a rugose appearance (44, 89). Furthermore, *C. pylori* can be differentiated from the spirochetes because the latter group possesses axial filaments which *C. pylori* lacks (89).

C. pylori can also be differentiated from *C. mustelae* which is a *Campylobacter*-like organism (CLO) isolated from normal and inflammed gastric mucosa of ferrets (34, 35, 36). Electron microscopic studies showed that unlike *C. pylori*, the flagella of CLOs are peritrichously arranged around the cell.

At the molecular level, SDS-PAGE reveals that *C. pylori* has a unique protein profile which differentiates it from other campylobacters. Cellular fatty acids are another taxonomic marker. Fatty acid analysis reveals that the major acids are tetradecanoic acid (14:0) and cis-9,10-methyleneoctadecanoic acid (19:0), with very small amounts of hexadecanoic acid (16:0) (7). In comparison, the major cellular fatty acids of true *Campylobacter* species are octadecanoic (18:1) and hexadecenoic acids (16:1) (44, 65). In addition, the fatty acid profile of *C. pylori* is distinct from that of spirochetes, vibrios, and other enterobacteria (44). Other biochemical differences that separate *C. pylori* from other campylobacters include the absence of the respiratory quinone-methylated menaquinone-6 in *C. pylori* but its presence in campylobacter species, as well as the presence of the enzymes urease, catalase and superoxide dismutase in *C. pylori*, which are absent in other campylobacters (61).

Another source of disparity between *C. pylori* and other campylobacters arises from partial 16S rRNA sequencing studies (68, 110, 120). These studies reveal that *Thiovulvum* species and *Wollinella succinogenes* are as closely related to the true campylobacters as *C. pylori* (120). However, comparison of five major taxonomic features (ultrastructure and morphology, cellular fatty acids, menaquinones, growth characteristics and enzyme capabilities) showed that *C. pylori* was markedly different from other bacteria in the

genus *Wolinella* (42). As a result, it was recently proposed that *C. pylori* be transferred to a new genus, *Helicobacter* (42). Further useage will decide whether the organism will be called *C. pylori* or *H. pylori.*

Epidemiology. Since the isolation of *C. pylori* seven years ago, evidence has rapidly accumulated that suggests there is a strong correlation between the presence of *C. pylori* and several gastrointestinal diseases including type B gastritis, duodenal ulceration, gastric ulceration and non-ulcer dyspepsia. The organism has been isolated from humans throughout the world, although minor variations exist within certain ethnic populations (10). The organism has been identified in 70-80% of patients undergoing gastroscopy (115). Of these patients, 80% had type B gastritis (12), 90-100% had duodenal ulcers (115), and over 70% had gastric ulcers or nonulcer dyspepsia (115). Although *C. pylori* has only recently come of interest, repeat studies of gastric biopsies taken ten years ago confirm that *C. pylori* was in fact present in the diseased stomach. *C. pylori* was not recognized earlier simply because pathologists did not look for bacteria in gastric samples (8). Indeed it was widely held that the stomach was normally bacteriologically sterile. The prevalence of the organism has been shown to increase proportionately with age, rarely being isolated from children (61). However, the most unsettling feature associated with the distribution of this bacteria is that it is found in 56% of the world's population over 40 years of age; although, always asociated with inflammed gastric tissue (45, 70). Because of this last statistic, it has been questioned whether *C. pylori* is simply a commensal of the human stomach or whether it is truly a pathogen (64). Because the prevalence of *C. pylori* increases with increasing age, questions are raised concerning the natural reservoir of the organism and its mode of

transmission. It is questions like these which perpetuate the controversy that surrounds *C. pylori*.

Natural reservoir. A survey of the literature reveals that investigators are undecided on this issue. One opinion is that the human stomach, lacking competitive flora, provides an ideal ecological niche for *C. pylori* (70). This opinion is based on epidemiological studies and in particular, the finding that over half the world's population over 40 years of age is colonized with this organism. The other point of view is that animals act as the natural reservoir, with the suspected mode of transmission being via the fecal-oral route. This opinion is based on the isolation of gastric *Campylobacter*-like organisms (CLOs) from the stomachs of *Rhesus* monkeys (105), baboons (70), ferrets (35), and cats (61, 71). In addition, infections established in gnotobiotic piglets showed that *C. pylori* does present with the active inflammation that is the hallmark of human infections (10, 57, 62). However, a recent report by Shames et. al. (123) states that a strain of *C. pylori* that was isolated from the stomach of one patient was also isolated from dental plaque from the same patient. Of interest is the fact that dental plaque is also the natural reservoir for *Wollinella* sp., the genus shown by 16S rRNA sequencing studies to be closely related to *C. pylori*.

Koch's postulates. In an attempt to assess whether there is a causal role for *C. pylori* in these gastric diseases (85) or whether *C. pylori* is simply a "...secondary invader in an already altered gastric mucosa..." (41), it is necessary to recall Koch's postulates (13):

1. The organism should always be found in animals suffering from the disease and should not be present in healthy individuals.
2. The organism must be cultivated in a pure culture away from the

animal

3. Such a culture, when inoculated into susceptible animals, should initiate the characteristic disease symptoms.

4. The organism should be reisolated from these experimental animals and cultured again in the laboratory, after which it should still be the same as the original organism.

Postulate 2 has been proven by the work of Warren and Marshall (12). In evaluating postulate 1, it is worth considering that 10% of patients who have *C. pylori* do not have gastritis or ulcers (12). Those in favor of *C. pylori* as a human pathogen point out that the pathogenesis of ulcers, as well as nonulcer dyspepsia, is unknown. Current theories favor the interaction of aggressive, endogenous factors such as stomach acid or pepsin, with aggressive, exogenous factors such as nonsteroidal antiinflammatory drugs or ethanol (11). Those favoring a pathogenic role for *C. pylori* in stomach disease consider the organism to be an endogendous factor. Opponents of a causal role for *C. pylori* remind us that "...an epidemiological association does not prove cause and effect" (142).

However the best evidence to date that *C. pylori* does play a role in causing gastritis comes from two volunteer challenge studies which irrefutably prove Koch's third postulate (102). The first study was performed by Marshall in 1985 (90). Before challenge, endoscopic biopsy confirmed *C. pylori* was absent and his stomach was rendered alkaline by administration of an H_2 antagonist. Within a few days of swallowing 10^9 organisms/mL, Marshall developed epigastric distension, malaise, halitosis, and vomiting. On day 10, endoscopic biopsy revealed the presence of *C. pylori* and active gastritis as evident from the infiltration of polymorphs. At

the end of two weeks, *C. pylori* was no longer present and the gastritis was clearing. The second volunteer challenge presented with a similar set of symptoms (102). In 1987, Morris swallowed 3×10^5 bacteria/mL of *C. pylori*. Three days later, Morris experienced moderate to severe episodes of epigastric pain. Five days later, *C. pylori* was cultured from antral biopsies which were accompanied by the infiltration of polymorphonuclear leukocytes. By day 11, *C. pylori* could be isolated from a fundal biopsy. Unlike the challenge by Marshall, the study by Morris resulted in a persistent infection characterized by a minimal but distinct chronic gastritis.

Pathogenesis. In order to assess the mechanisms by which *C. pylori* associates with the stomach, it is necessary to review the factors thought to be associated with its virulence (116).

One feature of the organism that makes it particularly well suited to the intestinal mucosa is its spiral shape. This property allows it to be mobile in a highly viscous environment (51). *In vitro* studies of motility in methyl cellulose reveal that *C. pylori* can move much faster than rod shaped bacilli such as *E. coli* (51). Of interest is the characteristic alignment of the organism such that it normally moves in parallel with mucous strands. Corkscrew motility, aided by the sheathed flagella, is another adaptation to this environment (70). Because *C. pylori* is sensitive to gastric acid (142), it is hypothesized that its flexibility and motility facilitate its movement into the mucous gel. This new location not only protects the organism from the acidic conditions found in the lumen but also provides it with a microaerophilic environment (142). It is then hypothesized that *C. pylori* is drawn by a chemotactic gradient that is diffusing out of gastric epithelial cells thus relocating *C. pylori* deeper into the gastric epithelium (61). This

hypothesis is supported by ultrastructural studies of biopsy specimens of the antrum of the human stomach which revealed that *C. pylori* is characteristically associated with gastric mucosal epithelial cells both at the apical surface and at intracellular junctions. Ultrastructural studies also reveal that *C. pylori* is often found in "cave-like" structures in the intestinal mucus. It is hypothesized that this niche is generated by extracellular proteolytic enzymes of *C. pylori* that degrade mucin, the protein comprising mucus (10, 142). The fact that there is decreased viscosity in the gastric mucus isolated from patients with chronic active gastritis (96), provides support for this hypothesis. Formation of cave-like structures by these proteases is a serious alteration of the gastric mucosa since the integrity of the mucous is required to protect the epithelium from damage by gastric acids. Exposure to gastric acids would lead to ulceration of the epithelium.

Once localized at its target cell, it is hypothesized that the colonization of *C. pylori* is mediated by specific adhesion mechanisms (103). The concept of an adhesion mechanism is supported by the observation that the surface of *C. pylori* is surrounded by a capsule-like network of a fibrillar colonization factor antigen (56). Studies have shown that this adhesin is actually a hemagglutinin (25), however the actual composition of the hemagglutinin is not known at this time. Chemical studies have shown that this hemagglutinin is specific for sulfated and sialic acid containing glycolipids having terminal lactosyl moieties (75) which are found on a variety of cell surfaces including erythrocytes. Suggested structures include GM3 ganglioside (124) and N-acetylneuraminyllactose (126). Serum antibody responses to the N-acetylneuraminyllactose-binding hemagglutinin showed that 81.5% of patients with ulcers tested positively for this antigen by ELISA

(28). *In vitro* adherence of *C. pylori* has been demonstrated with mouse Y-1 adrenal cells, which have N-acetylneuramyllactose on their surface (29). Adherence of *C. pylori* to these cells was blocked by neuraminidase and by a neuraminlactose-containing protein (30).

The specific attachment of *C. pylori* to its target cell is confirmed by ultrastructural studies which show that the microvilli at the tight junction complex are shortened (11). This modification of the host cell would allow the cell envelope of the bacterium to be right next to the surface of the mucous cell. This cell contact has occasionally been visualized as a thickening in the host cell creating a structure known as an adherence pedestal. This structural modification is also a feature of host cells as a result of infection by enteropathogenic *E. coli*. Following attachment of *C. pylori* at intestinal mucosal cells is damage to these cells by the production of cytotoxins. This results in the leakage of nutrients from these cells, specifically, urea. The reason that urea is thought to be of such importance to the growth of *C. pylori* is that this bacteria constitutively expresses a urease enzyme. The enzyme has recently been characterized and shown to be composed of three subunits (subunit Mrs of 66 kDa, 63 kDa, and 31 to 29 kDa) (23), have an optimal pH of 8.2, a pI of 5.9, and an optimal temperature of 45 C (31, 99). The *C. pylori* urease is 1000 times more active than the urease activity of *Proteus vulgaris* (50), ten times more active than the urease activity of urinary tract isolates such as *Klebsiella* species (99) and two times more active than the urease activity of *Proteus mirabilis* (99). "No organism would produce such an enzyme without a purpose" (142). Because *C. pylori* is nitrate negative but is glutamate dehydrogenase positive, it is

hypothesized that *C. pylori* utilizes the ammonia liberated from the breakdown of urea as a nitrogen souce (142).

In addition to the role of urease as an important nutrient for *C. pylori,* this enzyme is also proposed to play an important role in the pathogenesis of this organism. There are presently two theories to explain how urease acts as a virulence factor. The first hypothesis is proposed by Hazell and Lee (49). It states that the rapid hydrolysis of urea at intercellular junctions, with its concomittant release of ammonia, results in a rise in pH of the gastric epithelium. This alteration in pH disrupts the normal passage of hydrogen ions from the gastric glands, through the mucus, to the lumen of the stomach. The implication of this back diffusion of hydrogen ions is clearly a condition of hypochlorhydria or a very acidic stomach, which predisposes the stomach for ulcer formation (49). The mechanism for this hydrogen ion back diffusion is thought to be related to the Na^+/K^+-ATPase pump which is the driving force for the passage of hydrogen ions in to the lumen of the stomach. When the pH at the mucosal surface becomes alkaline, it is thought that the selectivity of this pump is altered such that sodium ions pass in to the lumen of the stomach, while hydrogen ions back diffuse.

The second theory put forth for how the urease enzyme of *C. pylori* acts as a virulence factor considers cytopathic effects induced on host cells by the high concentrations of ammonia liberated (5). *In vitro* studies have shown that the concentrations of ammonia generated by the urease enzyme of *C. pylori* have a direct cytopathic effect on Vero cells. The cells round up and then lyse after exposure to concentrations of ammonia as low 2.7 mmol/L at physiological pH. These investigators postulate that the ammonia generated by *C. pylori* is sufficient to produce cell damage that would result

in the chronic inflammation that is characteristically associated with a *C. pylori* infection.

In addition to the cytopathic effects of the urease activity of *C. pylori,* this bacteria can produce direct cytopathic effects on host cells. *C. pylori* possesses a proteinaceous, cytoxic activity that is present in broth culture filtrates. Exposure of this cytotoxin to mammalian cells causes the cells to undergo intracellular vacuolization, a nonlethal, but cytopathic effect (74). Sixty-six point six percent of *C. pylori* strains isolated from patients with peptic ulcer produced the toxic activity; 30.1% of *C. pylori* strains isolated from patients with chronic active gastritis possessed the cytoxic activity (32).

Immunology. The chronic inflammation that is the hallmark of *C. pylori* infections is the host's response to dealing with the cell damage that results from these cytopathic factors. The controversy surrounding the infiltration of lymphocytes and plasma cells (135) into the gastric mucosa is whether or not the colonization of *C. pylori* is instrumental in initiating this local immune response (21). It is pointed out that if *C. pylori* does initiate this response, then antibodies should be made at the site of infection and they should be "...in part specific for the organism" (135). In support of this proposal is the secretion of IgA and IgM antibodies by the gastric mucous cells into the lumen of the stomach. Immunohistochemical studies have shown that these antibodies are specific for *C. pylori.* Moreover, these studies have shown that the majority of organisms are localized at the mucosal surface, although some were also present in gastric pits. However those organisms located at the base of the gastric pit were not coated with immunoglobulins while those organisms located at the mucosal surface

were coated. This difference is no doubt due to the protection afforded by this site. The significance of this point will be emphasized later.

In addition to a local immune response against *C. pylori,* there is also a systemic response to the organism as *C. pylori* specific antibody (IgG) is found in the serum of colonized patients (141). Access of *C. pylori* to the systemic circulatory system is no doubt provided by the destruction of the gastric epithelium mediated by the virulence factors associated with *C. pylori.*

Treatment. Further evidence favoring a pathogenic role for *C. pylori* in the etiology of ulcers and gastritis is the disappearance of these pathologic conditions after treatment aimed at eradicating the organism (7). After successful treatment of gastritis with antibiotics administered along with colloidal bismuth subcitrate, histological studies revealed that infiltration of polymorphonuclear leukocytes had ceased and that *C. pylori* was no longer present (136, 137). In addition there was a decrease in IgG antibodies specific for *C. pylori* as determined in an ELISA assay using sonicated *C. pylori* cells as the antigen. This link between the resolution of gastritis and the disappearance of *C. pylori* is further substantiated by a study performed by Barry Marshall. He reported that ulcer healing occurred in 93% of patients cleared of *C. pylori* whereas the rate of ulcer healing was only 57% in patients who had a chronic *C. pylori* infection (93).

One of the main problems related to treatment of *C. pylori* associated gastric diseases is the high relapse rate of gastrointestinal symptoms. It has been shown that this relapse coincides with a reappearance of *C. pylori* (114). Langenberg and coworkers studied the restriction fragment polymorphisms (RFPs) of strains that reappeared following relapse (67). They showed that a relapse is most frequently due to a recrudescence of the same strain as

opposed to a new infection by another strain. This is not an unlikely phenomenon when it is recalled that at the base of the gastric pits reside *C. pylori* cells which are not coated with immunoglobulins and are thus protected from the local immune response. The work of Langenberg and coworkers is supported by immunoblot fingerprinting studies (15). Relapses in five out of eight patients were due to a recrudescence of the same isolate; relapses in the other three patients revealed a change in the immunoblot pattern. It is conceivable that these patients were infected with a new strain, or the existing strain was capable of producing multiple antigenic types (15).

Clearly the location of *C. pylori* at the base of gastric pits, a physically protected site, presents a problem when attempting to eradicate this organism. Ideally, the antibiotic of choice would be stable and active over a wide pH range, have local and systemic activity (93), and be able to penetrate the gastric mucosa through to the gastric pits (67, 96). *In vitro, C. pylori* has been shown to be sensitive to the β-lactams, aminoglycosides, macrolides, and nitrofurans and the new quinolones (7, 41, 43, 66). However, *in vivo,* only a few antibiotics: amoxicillin (108), nitrofurantoin, furazolidone, erythromycin, tinidazole, omeprazole, and oxfloxiacin have eradication rates greater than 10% (53, 67, 86). Another problem is the appearance of resistant strains. So far, *C. pylori* has been reported to develop resistance to the quinolones (92). In light of these difficulties, physicians have turned to using colloidal bismuth subcitrate (1) since this agent has been shown to have antibacterial properties with eradication rates of up to 30% to 40% for *C. pylori*.. Considering that bismuth salts were "...relegated to the role of an over-the-counter medicine for many years..." (89) its recent emergence as an

antimicrobial agent is surprising. Electron microscopy studies have shown that at an acidic pH, bismuth salts precipitate. Crystals of bismuth citrate and bismuth oxychloride bind to glycoproteins and as a result, may impair hydrogen ion back diffusion across the gastric mucosa. These crystals also coat the outside of *C. pylori* cells. As a result, the cells swell, lyse and are removed within 24 hours (93). It is this antimicrobial action of colloidal bismuth salts against *C. pylori* that explains the low relapse rates that are associated with its use. In contrast, high relapse rates are associated with the treatment of ulcers with H_2 antagonists because these agents lack the antimicrobial activity that colloidal bismuth salts have (9). Most frequently, colloidal bismuth sulfate is administered along with an amoxicillin, tinidazole, erythromycin, ofloxacin or tetracycline plus metronidazole (86). The advantage of a combined therapy is the synergistic activity (138) between the two agents resulting in eradication rates of 70%-90% (86).

Diagnostics. The method of choice for monitoring eradication of *C. pylori* is the ^{14}C-urea breath test. This test takes advantage of the strong urease activity possessed by *C. pylori* (87, 95). The basis for this test is the hydrolysis of urea that is labelled with an isotope of carbon and subsequent liberation of labelled carbon dioxide. Breath samples are obtained every 10 minutes for 190 minutes and analyzed by gas-isotope-ratio mass spectrometry if the label was the nonradioactive isotope, ^{13}C, or in a scintillation counter if the label was ^{14}C. The fact that this activity is not a property of mammalian cells and is not found with such a high activity in other microbes enhances its value as a predictor of viable *C. pylori* organisms (52).

In cases where treatment has failed to remove the organism and for initial diagnosis, especially in patients over 40 years of age in order to rule out possible malignancies (47), an invasive technique is required to diagnose the presence of *C. pylori*. Because *C. pylori* is unique in inhabiting the stomach and upper part of the duodenum, the necessary technique is fiberoptic endoscopy (95). Once obtained, the biopsy sample can be analyzed in several ways.

The first method of analysis is the rapid urease test which relies on the high urease activity associated with viable *C. pylori* cells (134). This test is performed by placing a crushed biopsy specimen into a few drops of urea medium and incubating the broth at 37 C. The resulting increase in pH from the liberation of ammonia causes a change in the colour of the indicator. The extent of colour change is noted at 1 hr, 4 hr, and at 24 hr (95). A modification of this procedure has been developed to allow for detection of *C. pylori* within one minute of obtaining the biopsy in the endoscopy room (3). This one minute biopsy test has correctly predicted the presence of *C. pylori* in 19/21 patients from which the organism was later isolated. Of course, factors that must be considered for all diagnostic tests include: sensitivity, specificity, speed, simplicity, cost, and globality (95). The rapid urease test is consistent with a specificity of 100% and a sensitivity that ranges from 83% to 91% (49). One factor that may affect the sensitivity is the sampling of other urease positive organisms such as *Klebsiella* sp. or *Proteus* sp. which may be present in the stomach as a result of a reflux disorder (88). The disadvantage of this test is the large numbers of organisms that are required for a positive result.

From the gastric juice obtained with endoscopy, the concentration of urea can also be a predictor of viable *C. pylori* organisms, although attempts at culturing the organism directly from gastric juice have been unsuccessful. No doubt this failure is related to the sensitivity of *C. pylori* to acid (95). Culturing *C. pylori* from biopsy specimens is however the method that provides maximum sensitivity with maximum specificity. This is because only one bacterium plated from the specimen will yield a pure culture (95) that can be positively identified (109). Unfortunately, use of bacteriology as the reference technique is not 100% reliable at the present time. This is because the organism is delicate, fastidious and a slow grower. A reliable transport medium has not been developed and the culture media needs refinement (95). Selective agents are required to inhibit growth of normal flora from other sites contaminating the biopsy such as *Streptococcus* sp. and *Pseudomonas* sp.

Because of this problem with viability, investigators rely or microscopic examination of the biopsy (6, 83, 95). Organisms in smears have been reliably identified with the Gram's stain (100), the Wright's stain and acridine orange (95). However,the sensitivity of these detection methods is decreased if there is a patchy distribution of the organism. Increased specificity has been reported with an indirect immunofluorescence assay using polyclonal or better still, monoclonal antibodies, specific for *C. pylori* (26). The obvious advantage of using monoclonal antibodies is that other contaminating gram-negative rods will not be falsely identified as *C. pylori* (32). Monoclonal antibodies can also be used in conjunction with immunofluorescence or immunoenzymatic techniques for examination of tissue specimens. The advantages of these techniques are that they provide

the investigator with information concerning the relationship of the organism with the gastric mucosa. Other stains used to examine fixed biopsy specimens include: hematoxylin, eosin, and Warthin-Starry (83). This last stain has the greatest contrast, but the disadvantage of all of these stains is the expertise that is required to interpret them.

The other method, apart from culturing, that provides a very high level of specificity is hybridization of genomic DNA with specific probes labelled with ^{32}P (6, 101) or biotin (139). Although this technique demands that there be little contamination from other bacteria, this requirement is not a problem when diagnosing *C. pylori* considering specimens are taken from the stomach. The advantage of DNA hybridization over DNA restriction endonuclease analysis is that the latter method produces a complex digestion pattern thay may hide subtle but significant differences (84). The other disadvantage of restriction endonuclease analysis is that the genomes of up to one third of *Campylobacter species* are known to possess plasmids (119). Plasmid DNA may complicate an already complex pattern.

While the above methods are invaluable for diagnosing and evaluating the treatment of individual patients, they are not practical for screening large numbers of patients as is required for epidemiological studies (98). The test most commonly employed for this purpose is the enzyme-linked immunosorbent assay (ELISA) (104, 122, 140). While this test is reliable, inexpensive and easy to perform, its application to *C. pylori* presents some difficulties. One of the problems is related to the fact that colonization with *C. pylori* usually presents as a persistent and chronic infection. In terms of antibody titres, this means that there will not be a sudden rise or drop in antibody concentrations which is one of the features

of an infection that the ELISA assay relies on. Studies have confirmed that IgA titres remain high while IgG titres do drop but they do so gradually (52). The other problem is that assays performed using sonicated whole cells as the antigen have low sensitivity because the amount of antigen per sample is very low. Specificity is also inadequate because of cross reactivity reported with other species of campylobacter (98). Central to refining immunological assays is the determination of species specific, strain specific and cross reactive antigens on the surface of *C. pylori*.

Surface antigens. Initial studies of surface antigens have focused on protein and LPS profiles. Analysis of the LPS by SDS-PAGE has revealed strains which produce smooth, rough, and semi-rough LPS. Immunoblotting studies revealed that the core region of the *C. pylori* strains studied is cross reactive with the core regions of *C. jejuni* and *C. fetus*. This finding favors the hypothesis that among gram-negative bacteria there exist similarities in the antigenic structure of lipid A (112).

Protein analysis by SDS-PAGE reveals that *C. pylori* contains seven major proteins. Perez-Perez and Blaser report that these proteins have subunit Mr's (kDa) of 62, 56, 53, 48, 29, and 26 (112). In addition, one group of investigators has reported the presence of another protein at 120 kDa (2). In the case of certain common surface protein antigens of *C. pylori*, cross reactivity with other *Campylobacter* species has been demonstrated with immunoblotting. Using rabbit antiserum specific for the flagellin of *C. jejuni*, Lee and coworkers (72) showed that antigenic cross reactivity among *C. pylori, C. jejuni* and *C. coli* occurred with a 57-59 kDa protein. Mills and coworkers (97) have extended this cross reactivity to include *C. laridis, C. upsaliensis*, and *C. sputorum*. However, they note that the Mr of the common

antigen that cross reacts with antiserum specific for the 62 kDa flagellin protein of *C. jejuni* varies in Mr from 53 to 64 kDa in different *C. pylori* strains.

Flagella. The bacterial flagellum is a locomotive organelle which is composed of at least three elements: a basal body or rotary motor, a hook or universal joint, and a filament or propellor (82). Flagella are important virulence factors in certain infections. Studies with *Vibrio cholerae* have shown that they are thought to act as adhesins (4). Studies with *C. jejuni* using anti-flagellar antibodies showed that motile strains are necessary for infection in mice (107, 131). This finding may be related to the observation that mucus colonization is a major determinant of the pathogenicity of *C. jejuni* (73) and that the flagella facilitate movement of the bacterium to this location. Thus it appears that there is a dual role for the flagellum in promoting infection: as an adhesin and as a motility organelle. However, a recent report suggests that flagella may be linked to a third role in promoting infection. Previous experiments using the burned mouse model showed that nonmotile, nonchemotactic strains of *S. typhimurium* (16) and *P. aeruginosa* (22) are avirulent. Recently, it has been suggested that this lack of virulence is not due to mutations in the genes encoding flagellin as such, but in a virulence gene(s) that extends into the region encoding the biosynthesis of flagella (17).

Most of the flagellar filament is constructed from one protein called flagellin (82). Assembly of the flagellar filament must be engineered so that the base of the flagellum is aligned with surface components of the bacterium. Electron microscopy studies have revealed that the overall assembly process proceeds from the proximal end, with the basal body and

hook proteins, toward the distal end with the flagellar filament. Flagellin that is synthesized actually passes up through the core of the filament and is added to the growing distal end of the filament. The basal body is embedded in the layers of the cell surface. It consists of the rod and four sets of rings. The inner two rings, S and M, are in the planes of the cell membrane. The next ring, P, is in the plane of the peptidoglycan layer while the outermost ring, L, is in the plane of the outer membrane. In *S. typhimurium*, at least seven basal body proteins have been identified, although as many as 15 may be present. It is not presently known which components of the basal body rotate and which are stationary (82).

The hook connects the basal body with the flagellar filament. Although the hook is structurally similar to the filament, it is composed of a separate hook protein which has a Mr of 42 kDa. Its length does not vary among isolates and is usually 80 nm. Antigenically, the hook is similar in *E. coli* and *S. typhimurium* isolates.

The flagellar filament ranges in length from 5 to 10 μm and has a constant diameter of 20 nm (82). Ultrastructural studies of the flagella of *C. pylori* reveal that the flagella are 3 to 5 μm long and that the flagellar filament has a diameter of 9 to 12 nm. Electron microscopy studies have revealed that flagellar filaments from different 'H', or flagellar serotypes of *E. coli*, have corresponding and different flagellar surface patterns, even though the flagella from each strain appear structurally identical (69). Lawn and coworkers have described six such morphotypes for *E. coli* (A through F). Similar studies have revealed that two broad classifications of flagellar filaments exist among bacteria: plain filaments and complex filaments (113). Plain filaments have a smooth surface structure, can alternate between

clockwise and counterclockwise rotation and are found in most free-swimming bacteria such as *E. coli, S. typhimurium,* and *B. subtilis.* The surface of complex filaments have a helical pattern of alternating ridges and grooves, and can rotate only in the counterclockwise direction. It is thought that these rigid grooves confer upon the filament the rigidity it needs in order to move through a viscous environment. So far complex filaments have been have been isolated from only three bacteria, all found in soil (113).

The surface of the flagellar filament of *C. pylori* is distinct in that it is covered by a sheath. The presence of this sheath is consistent with flagellar morphotypes E and F as described by Lawn and coworkers (69). The presence of flagellar sheaths have also been reported for gram-negative and gram-positive bacteria including: *Vibrio cholerae, Vibrio metchnikovii, Bdellovibrio bacteriovorus , Pseudomonas stizolobii* and *Bacillus brevis* (38).

There is disagreement among investigators regarding the biochemical composition of the flagella sheath. Hranitszky and coworkers (55) report that the sheath of *Vibrio cholerae* contained three proteins of Mr (kDa) 61.5, 60.0, and 56.5. They further showed that antibody specific for LPS labelled the cell but not the sheathed flagellum. In contrast, Fuerst and Perry (37) recently reported that monoclonal antibodies with group and type specificity for LPS antigens labelled the sheath of the flagellar filament as well as the cell. This result suggests that the sheath of the vibrio flagellar filament is a continuation of the outer membrane of the cell. This theory is supported by the findings of Glauert et. al. (40) through electron microscopic studies of *Vibrio metchnikovii.* Another group supports this finding by reporting that the sheath contains 57% more phospholipid and 26% less protein than that

which is found in outer membranes (127). They predict that this unique balance of components would produce a flexible, fluid-membranous structure which would be necessary in order for the filament to rotate.

The flagellar filament itself is composed of flagellin monomers that range in Mr from 62 kDa for *C. jejuni*, 45-53 kDa for *P. aeruginosa*, 48-58 kDa for *Salmonella* sp., and 51-57 kDa for *E. coli* (38). Amino acid compositions of the flagellins differ, but they have some similarities. For instance, both tryptophan and cysteine residues are absent, while both proline and histidine residues are present in small quantities or are absent (79). Some flagellins have the unusual characteristic of containing methyl-modified residues; specifically N-methyl-lysines (82). Flagellin also has the unusual property of being able to self-polymerize into its original filament structure. This property is reversible and is utilized in purification schemes where heat or acid pH are used to depolymerize flagellar filaments into their constituent flagellin monomers (82).

It was previously thought that all filaments were polymers of a single monomer as is the case for *E. coli*, but recent evidence has suggested that the filament can be composed of more than one type of flagellin protein. For instance, the filament of the soil bacterium, *Rhizobium melioti* is assembled from heterodimers of two related flagellin subunits (113). The filament of *Caulobacter crescentus* is assembled from three different flagellins (subunit Mrs 29.0, 27.5, and 25.0 kDa) (129). The 25 kDa or flagellin A protein constitutes the majority of the filament while the other two flagellin proteins are concentrated in the proximal end of the filament near the hook. Purified flagellar filaments from *Halobacterium halobium* also contain

three different flagellins, and these were characterized as sulfated glycoproteins (39).

The flagellar filaments of *S. typhimurium*, *C. coli*, and *C. jejuni* are unusual in that they exhibit antigenic variation (46, 48). At different positions on its chromosome, *S. typhimurium* has two genes that code for two flagellin proteins, *H1* and *H2* (82). "The genes are highly homologous, but not identical, and so their products are antigenically distinct" (82). Each of these cells has flagellar filaments composed of only one type of flagellin monomer at a time. At a frequency of once every 10^3 to 10^5 generations, the non-expressed flagellin gene is expressed and vice versa (48). The mechanism for this transition is dependent on the inversion of a 970 base-pair region that is upstream from the *H2* gene and is bounded on either side by an inverted repeat. This invertable region contains the promoter for the *H2* region so that in one orientation, it is in phase for transcription of the *H2* gene. In the other orientation, transcription cannot proceed. Also contained in the *H2* region is the *rhi* gene whose product represses transcription of the *H1* operon. The *H2* operon also contains the *hin* gene, the product of which is required for the inversion event (82).

In *C. coli*, two antigenically different flagellins are encoded. Phase 1 corresponds to a flagellin of 61.5 kDa; phase 2 corresponds to a flagellin of 59.5 kDa (48). The frequency of phase 1 to phase 2 transitions is 2.0×10^{-5} per cell per generation; the frequency of phase 2 to phase 1 transitions is 1.2×10^{-6} per cell per generation. In addition to antigenic variation, *C. jejuni* exhibits phase variation or an on-off switching of flagella. However, "the relationship between phase and antigenic variation needs to be better elucidated" (46).

Recent three dimensional reconstruction of flagellar filaments from electron micrographs provide insight in to how antigenic changes could be accomodated in the structure of the filament (129). Three dimensional reconstructions of the filament of *S. typhiumurium* have shown that flagellar subunits are arranged with the same helical packing such that 11 axial rows of subunits are generated forming outer knobs and inner loops. The three dimensional structure of *C. crescentus* flagellar filaments differs from that of *S. typhimurium* in that the outer knobs are absent in *C. crescentus*. This finding is supported by the smooth and narrow appearance of the filaments of *C. crescentus*. Amino acid sequence alignment using the FASTP program reveals that the N and C-terminal ends of the *C. crescentus* flagellin sequence are homologous to the N and C-terminal ends of the *S. typhimurium* sequence. However, when the central region of the flagellin of *S. typhimurium,* corresponding to amino acids 160-396, is used as the query sequence, neither the flagellin of *C. crescentus* nor the flagellin of *B. subtilis* are aligned. Considering that all proteins are flagellin, this apparent anomaly can be explained in terms of differences in molecular weight. The Mr of the flagellin of *C. crescentus* is approximately one half the Mr of the flagellin of *S. typhimurium.* Therefore, from sequence data and Mr's, it can be deduced that the central region of the flagellin of *S. typhiumurium* is absent in the flagellin of *C. crescentus*. The findings of Trachtenberg and DeRosier are consistent with findings of Wei and Joys (132). The latter group reported that the N and C-terminal portions of flagellins are more conserved compared to the rest of the sequence. The same authors also showed that the regions responsible for antigenic differences in the *d* and *i* serotypes of *S. typhimurium* flagellin could be localized to the central region

of the protein, corresponding to amino acids 160 to 396. It is interesting to note that a recent report by Smith and Selander (125) is in direct contrast to the findings of Wei and Joys. The report states that the central sequence from seven strains of *S. typhimurium* were identical instead of variable.

In an attempt to correlate the findings of Wei and Joys with a three dimensional reconstruction of the filament of *C. crescentus,* Trachtenberg and DeRosier (129) state the the absence of the outer knobs in *C. crescentus* and the concomitant absence of the central region of amino acids, suggests that the antigenically variable region forms a loop or outer knob, while the amino and carboxy termini are directed inward. Inherent in this reconstruction is the fact that this antigenically variable, outer knob is not required for motility. It is this central region then that allows for the antigenic variation that is exhibited by *S. typhimurium.*

A similar model may be in order for *Campylobacter* in that the N-terminals of flagellin from both phases of *C. coli* (78) and four strains of *C. jejuni* (79, 107) are homologous with those sequences reported for *S. typhimurium* (60) and *B. subtilis* (20)

In light of the reports of a common antigen among *Campylobacter* species, including the 58 kDa protein of *C. pylori,* it would be interesting to determine where these cross reactive sites are located in this three dimensional reconstruction of the flagellar filament. A first step toward this end is determining the identity of the 58 kDa protein of *C. pylori* and studying its cross reactivity with other *Campyobacter* species.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All stock cultures used in this study were maintained at -70 C in 15% (v/v) glycerol in Trypticase Soy Broth (Gibco Laboratories Inc., Grand Island, NY). Three *C. pylori* strains were used in the study: 5442, 5155, and 5294. These strains were isolated in Australia by A. Lee (School of Microbiology, University of New South Wales) from gastric biopsies of patients suffering gastritis. Cultures were grown at 37 C for 48-72 hr in an atmosphere containing 10% CO_2 and 5% O_2 that was generated by a *Campylobacter* gas pack (Oxoid Ltd., Basingstoke, United Kingdom). Cultures were routinely streaked on chocolate Mueller-Hinton agar plates (Gibco) which were supplemented with Campylobacter antibiotics (0.001% (w/v) vancomycin, 0.005% w/v trimethoprim, 0.0075% w/v colymicin (Sigma Chemical Co., St. Louis, MO) For electron microscopy work, cells were grown for 72 hr in Brain Heart Infusion Broth (Gibco) supplemented with 10% horse serum (Gibco) and 0.25% yeast extract (Gibco) (H. Lior, personal communication) in a microaerophilic environment at 37 C. Cultures of *Campylobacter coli* VC167B, *Campylobacter jejuni* VC74, *Campylobacter laridis* and *Campylobacter fetus* VC78, obtained from the University of Victoria *Campylobacter* collection, were also grown on chocolate Mueller-Hinton agar plates supplemented with Campylobacter antibiotics. Plates were incubated for 24 hr in a Forma model 3185 incubator (Marietta, OH) at 37 C with a CO_2 concentration of 10%. Cultures of *Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus* species, *Proteus vulgaris, Salmonella typhimurium, Salmonella enteriditis, Aeromonas hydrophila* TF7 and *Escherichia coli* were grown on Trypticase Soy Agar (Gibco) plates and incubated in air at 37 C for 24 hr.

Biotyping. Biochemical identification tests were performed according to the biotyping scheme outlined by McNulty and Dent (94).

Cytochrome oxidase. To test for the presence of this iron-containing porphyrin enzyme (33), a loopful of bacteria was smeared onto a filter paper square 5 cm x 5 cm. One drop of 1% (w/v) tetramethyl-p-phenylenediamine dihydrochloride (Sigma) was added. A negative reaction was recorded as a pale yellow colour or no colour change while a positive reaction was recorded as the immediate appearance of a purple colour. *P. aeruginosa* was used as a positive control; *E. coli* was used as a negative control.

Catalase. The ability to convert hydrogen peroxide to water and oxygen through the action of the enzyme, catalase, was tested by adding one drop of 3% (v/v) hydrogen peroxide (Sigma) to a loopful of freshly grown bacteria smeared onto a slide. A positive reaction was recorded as immediate, intense bubbling of oxygen; a negative reaction was recorded as absence of bubbling or appearance of only a few bubbles of oxygen. S. *aureus* was used as the positive control; *Streptococcus* species was used as the negative control.

Alkaline phosphatase. To test for the presence of the enzyme alkaline phosphatase, the substrate, p-nitrophenyl phosphate disodium hexahydrate (Sigma) was prepared according to the protocol outlined by Lior et. al. (77). A loopful of freshly grown cells was suspended in 0.3 mL of 0.85% (w/v) NaCl. This suspension was then transferred to a tube containing the phosphatase substrate. The tube was incubated in air at 37 C for 2 hr. A distinct yellow colour was recorded as a positive reaction as a result of the hydrolysis of the substrate into p-nitrophenol; no change in

colour was recorded as a negative reaction. *C. jejuni* biotype 2 was used as the positive control; *C. jejuni* biotype 1 was used as the negative control.

Hippurate hydrolysis. To test for the ability to hydrolyze sodium hippurate, a loopful of freshly grown cells was emulsified in a tube containing 1% sodium hippurate and incubated, with frequent mixing, for 2 hr in a 37 C water bath. After incubation, 0.2 mL of a 3.5% (w/v) solution of ninhydrin (Sigma) was slowly overlaid on the sides of the tube. Without mixing, the tubes were returned to the water bath for another 10 min then examined for colour development. One of the products of the hydrolysis is serine which can be deaminated by ninhydrin, which is reduced to a purple-coloured product (33). No change in colour was recorded as a negative reaction. Group B *Streptococcus* was used as a positive control; *C. coli* was used as a negative control.

Urease. To test for the ability to hydrolyze urea into ammonia, water and carbon dioxide, a sterile swab was moistened with urea broth (33) and then smeared on top of a slide that was freshly smeared with cells. Immediate formation of a deep pink colour on the swab was recorded as a positive reaction since the alkaline end products cause the indicator phenol red to change from yellow to pink. Absence of colour development was recorded as a negative reaction. *P. vulgaris* was used as the positive control; a *Campylobacter* species was used as the negative control.

Rapid H_2S test. The ability to form hydrogen sulfide was tested in Brucella broth (Gibco) that was supplemented with ferrous sulfate and sodium metabisulfite according to the procedure outlined by Roop et. al. (121). A large loopful of freshly grown cells was gently suspended in the upper one third of the semisolid media and then the tubes were incubated

overnight at 37 C. Appearance of a black precipitate around the bacterial mass was recorded as a positive reaction; no color change was recorded as a negative reaction. *C. laridis* was used as a positive control; *C. fetus* was used as a negative control.

DNAse. To test for the presence of extracellular deoxyribonucleases as outlined by Lior et. al. (76), prepared agar plates supplemented with sodium salt of deoxyribonucleic acid and the dye toluidine blue (Prepared Media Laboratories, Richmond, BC) were inoculated with freshly grown cells by making a heavy streak line 3 cm long. The plates were incubated overnight in a microaerophilic environment at 37 C. A positive reaction was recorded as pink color development around the streak line; a negative reaction was recorded as no color change. *S. aureus* was used as a positive control; *C. jejuni* was used as a negative control.

Electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a mini-slab gel apparatus (Hoeffer Scientific Instruments, San Francisco, CA) by the method of Laemmli (63). Protein solubilized in sample buffer (10% glycerol (v/v), 5% 2-mercaptoethanol (v/v), 3% SDS (w/v), 0.1% (v/v) bromophenol blue in 0.0625 M Tris-HCl, pH 6.8) was stacked in 4.5% acrylamide (100 volts, constant voltage for 15 min) and separated with 12.5% or 7.5% acrylamide (200 volts, constant voltage for 30 min). Gels were stained for protein overnight with 0.25% (v/v) Coomassie Brilliant Blue R-250 (Serva Fine Chemicals, Heidelberg, West Germany) and then decolorized for 3 hr with 20% (v/v) ethanol-10% (v/v) acetic acid.

Silver staining of LPS. The presence of LPS was determined by a modification of the procedure of Hitchcock and Brown (54). Samples were

boiled for 10 min in 100 μL of SDS-PAGE solubilization buffer, an equal volume of 1 mg/mL Proteinase K (Sigma) was added, and proteins were digested for 1 hr at 60 C. After SDS-PAGE, gels were stained for LPS by the silver staining method of Tsai and Frasch (130) briefly outlined here. After fixing LPS overnight in 40% (v/v) ethanol-5% (v/v) acetic acid, LPS was oxidized for 5 min in the fixing solution plus 0.7% (v/v) periodic acid and then stained with silver (Two mL of concentrated NH_4OH was added to 28 mL of 0.1 M NaOH and while stirring, 5 mL of 20% (v/v) silver nitrate was slowly added. The final volume of 150 mL was made up in distilled water. For colour development, gels were placed in developing solution for 5 min (0.005% (w/v) citric acid plus 1.35% formaldehyde (v/v) in distilled water).

Isolation of outer membranes. The procedure described by Logan and Trust (81) was followed. One gram (wet weight) of washed cells was suspended in 10 mL of 20 mM TRIS, pH 7.4. A pinch of DNase and RNase (Boehringer Mannheim, Mannheim, West Germany) were added, and the cell suspension was passed three times through a French press at 16,000 lb/in^2. Intact cells were removed by centrifugation twice at 3,000 x g at 4 C for 30 min in a Beckman model J2-21 centrifuge (Beckman Instruments Inc., Palo Alto, CA). The total cell membrane fraction was then collected by centrifugation at 43,000 x g at 4 C for 30 min. Cell membranes were suspended in the TRIS buffer to give a concentration of 1 mg/mL of cell membrane preparation. Outer membranes were prepared by differential solubilization of the inner membrane by using the detergent sodium lauryl sarcosinate (30% solution; Grace & Co., Nashua, NH). The total membrane protein detergent ratio was 1% (w/v). After shaking for 30 min at room temperature, the outer membrane fraction was collected by centrifugation at

43,000 x g for 30 min at 4 C, and the pellet was washed three times in TRIS buffer.

Glycine extraction. This procedure was performed by the method of McCoy et al. (91). Cells from 20 plates (15 mm x 100 mm) of confluent growth were harvested, washed twice in distilled water, 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. Whole cells were removed by centrifugation at 10,000 x g for 20 min. The supernatant was neutralized with NaOH, and cellular debris was removed by filtration through a 0.45 μm filter (Millipore Corp., Bedford, MA). The preparation was then dialyzed twice overnight against distilled water, lyophilized, and stored at -20 C.

CsCl gradient ultracentrifugation. The protocol outlined by Chandler and Gulasekharam (18) was essentially followed. 43 plates (100 mm x 14 mm) of confluent growth of *C. pylori* 5294 were harvested in 80 mL of distilled water. The suspension was then homogenized for 3 min in a bead beater (Biospec Products, Bartlesville, OK). In order to free sheared flagellar filaments that may have become enmeshed in the cells, the cells were dispersed by vortexing for 3 min in ten times the culture volume in a solution containing 0.4% (w/v) NaCl, 0.02% (w/v) SDS, and 0.01% (w/v) thiomerosal. Deflagellated cells were removed by centrifugation at 16,000 x g for 20 min, and flagellar filaments were recovered from the supernatant by filtration through a 0.22 μm membrane filter (Millipore Corp., Bedford, MA). The flagella were then resuspended from the surface of the membrane in 0.4 % (w/v) NaCl by gently moving a glass rod across the surface of the membrane. This crude flagellar suspension was then

incubated with 1 mg/mL trypsin, and 0.5 mg/mL ribonuclease (Boehringer Mannheim) in 0.05 M phosphate buffer, pH 7.6 for 3 hr at 37 C. Final purification was achieved by CsCl equilibrium density gradient centrifugation. The flagellar suspension was mixed with CsCl (Pharmacia Fine Chemicals, Uppsala, Sweden) to give a final density of 1.3 g/cm^3 and centrifuged in a swinging bucket rotor at 182,000 x g for 20 h in a Beckman model L8-70M ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA). The flagella formed a dense white band in the central region of the centrifuge tube which was recovered using a needle and syringe. The CsCl was removed by filtration through an Amicon PM 10 ultrafiltration membrane (Amicon Corp. Danvers, MA). The flagellar filaments that were retained on the membrane were resuspended in distilled water and stored at -20 C.

Isolation of flagellin by ultracentrifugation-acid disassociation-differential centrifugation. As outlined by Logan and Trust (81), 73 plates (100 mm x 15 mm) of confluent growth of *C. pylori* 5294 were harvested in 50 mL of distilled water and homogenized twice for 30 sec in a bead blender (Biospec Products). Cells were pelleted by centrifugation at 10,000 x g for 20 min at 4 C and the pellet retained. The cells were resuspended in distilled water, pelleted by centrifugation at 10,000 x g for 20 min and the supernatant retained. The supernatants were then pooled and centrifuged at 100,000 x g for 1 hr at 4 C. The pellet was suspended in distilled water, adjusted to pH 2.0 with HCl and held at 0 C for 15 min. Material insoluble at pH 2.0 was removed by ultra-centrifugation at 100,000 x g for 1 hr. The supernatant was then adjusted to pH 7.0 with NaOH and left at 0 C for 30 min. The sample was then stored at -20 C.

Gel filtration. Flagellin isolated by the acid disassociation-differential centrifugation method of Logan and Trust (81) was concentrated five times in a Speedvac concentrator (Savant Instruments, Inc., Hicksville, NY) and centrifuged 5 min in a microcentrifuge at 12,000 x g to remove insoluble material. A 100 µL volume of the supernatant, containing 194 µg of protein, was injected onto a Superose 12 sizing column (10 x 300 mm) (Pharmacia). Protein was eluted from the column with 0.1 M NaCl in 20 mM TRIS, pH 7.4 at a flow rate of 0.5 mL/min for 45 min. Peak fractions were monitored at 230 nm, collected and dialyzed twice overnight against 4 L of distilled water using a Spectra/Por dialysis membrane (Spectrum Medical Industries, Los Angeles, CA), with a Mr 12,000-14,000 Dalton exclusion limit. Purification was monitored by SDS-PAGE gels stained with Coomassie blue as previously described.

Ion exchange chromatography. Flagellin isolated by the acid disassociation-differential centrifugation method of Logan and Trust (81) was concentrated and centrifuged 5 min in a microcentrifuge at 12,000 x g to remove insoluble material. A 500 µL volume of the supernatant, containing 194 µg of protein was injected onto a Mono Q anion exchange column (5 x 50 mm) (Pharmacia). The column was run isocratically at 0.5 mL/min for 10 min in 10 mM TRIS, pH 6.2 and then a gradient from 0-10% 1 M NaCl in 10 mM TRIS, pH 6.2 was applied to the column for 5 min. The column was run isocratically at this concentration of NaCl for 10 min during which time the protein of interest eluted as a single major peak. Peak fractions were monitored at 214 nm, collected, and dialyzed twice overnight against 4 L of distilled water using a microdialysis system with a prepared dialysis membrane (Bethesda Research Laboratories, Gaithersburg,

MD) having a Mr exclusion limit of 12,000-14,000 daltons. Purification was monitored by SDS-PAGE.

Isoelectric focusing. The isoelectric point of flagellin was determined using a vertical polyacrylamide mini gel system described by Robertson et. al. (118) using ampholytes in the pH range 3-10 (Bio-Rad, Richmond, CA). The gel mixture consisting of 0.6% (w/v) acrylamide plus ampholytes was poured into a mini gel apparatus (Hoeffer Scientific Instruments, San Francisco, CA) at a thickness of 1.5 mm and allowed to polymerize for 1 hr. 10 μg of purified flagellin isolated by the CsCl gradient centrifugation method was mixed with an equal volume of 60% (v/v) glycerol and 4% (v/v) ampholytes of the same pH range used to prepare the gel. A 5 μL volume of prepared protein isoelectric point standards in the same pH range (Bio-Rad, Richmond, CA) was also analyzed. The cathode solution was 25 mM NaOH; the anode solution was 20 mM acetic acid. Electrophoresis was performed at 4 C for 1.5 hr at 200 volts (constant voltage), then increased to 400 volts (constant voltage) for an additional 1.5 hr. After electrophoresis, protein in the gel was fixed for 30 min in 10% (v/v) trichloroacetic acid. The gel was then stained overnight with Coomassie blue (27% (v/v) isopropanol, 10% (v/v) glacial acetic acid, 0.04% (w/v) Coomassie blue, 0.5% (w/v) $CuSO_4$ in distilled water) and then destained with Destaining Solution (12% (v/v) isopropanol, 7% (v/v) glacial acetic acid, 0.5% (w/v) $CuSO_4$ in distilled water). The isoelectric point of flagellin was then extrapolated from the standard curve of the Bio-Rad protein standards at pH 3-10.

N terminal amino acid sequence analysis.

Electroblotting to Immobilon. 560 pM of purified 58 kDa flagellin was electrophoresed on SDS-PAGE using 0.5 mM sodium thioglycollate diluted 1:5 in running buffer as the anode solution. After electrophoresis, the gel was soaked in Transfer Buffer (10 mM CAPS Buffer, pH 11.0 + 10% (v/v) methanol) for 15 min. The Immobilon membrane (Millipore Corp. Bedford, MA) was prepared essentially as described by Szewczyk and Sumers (126). The membrane was soaked in 100 % methanol for 3 sec, rinsed in distilled water for 5 min, and equilibrated in Transfer Buffer for 15 min. Protein in the gel was transferred to the Immobilon membrane at 400 mA for 2 hr using a Biorad Transblot apparatus model 160/1.6 (Bio-Rad, Richmond, CA). After transfer, the Immobilon membrane was rinsed in water for 5 min and then stained with 0.5% (w/v) Coomassie blue for 30 min. The membrane was then destained with 50% (v/v) methanol-10% (v/v) acetic acid for 10 min, rinsed in distilled water for 10 min, and air dried on a piece of Whatman filter paper. The protein band of interest was excised from the membrane and stored in a microcentrifuge tube at -20 C.

Sequencing. The Immobilon membrane containing 560 pM of flagellin was inserted into an Applied Biosystems Model 470 sequencer (Applied Biosystems, Foster City, CA) and analyzed using the standard program with an on-line phenylthiohydantoin analyzer.

Amino acid composition analysis. 233 pM of purified flagellin was electroblotted to Immobilon, hydrolyzed by 6N HCl vapors, and analyzed for

amino acid composition in an Applied Biosystems Model 420 analyzer (Applied Biosystems, Foster City, CA).

Cysteine recovery. The cysteine content of flagellin was determined by pyridylethylating potential cysteine residues (24). To five microcentrifuge tubes, each containing 10 µg of purified protein, 50 µL of Denaturing buffer was added (1M TRIS, pH 8.5 containing 4 mM EDTA and 8 M guanidine-HCl mixed in a 1:3 ratio). 2.5 µL of 2-mercaptoethanol diluted 1:10 in distilled water was added to each tube. The tubes were mixed and incubated in the dark for 2 hr at room temperature under argon. At the end of this incubation period, 2 µL of 98% 4-vinyl pyridine (Aldrich Chemical Co., Milwaukee, WI) was added to each tube. The tubes were again mixed and incubated in the dark for 2 hr at room temperature under argon. At the completion of this incubation period, the contents of the five tubes were desalted by dialyzing twice overnight against distilled water using Spectra/Por dialysis tubing (Spectrum Medical Industries) 6.4 mm diameter with a Mr cut off of 12,000-14,000 Daltons . The sample was then lyophilized, run on SDS-PAGE and electroblotted to Immobilon (Millipore Corp.). Bovine serum albumin (BSA) (Sigma) was used as a positive control and flagellin from *C. coli* VC167B was a negative control. In addition, 100 pM of pyridylethylated cysteine was included as a standard.

Tryptophan recovery. In order to determine the tryptophan content of the flagellin, oxidation of these residues was prevented by reaction with vapors of 5% thioglycollic acid (143). 50 µg of purified protein was pyridylethylated and electroblotted onto Immobilon. The protein sample was hydrolyzed by the vapors of 6N HCl and 5% (w/v) thioglycollic acid and then analyzed for tryptophan. BSA was used as the positive

control, the flagellin protein from *C. coli* VC167B was used as the negative control. 100 pM of tryptophan was included as a standard.

Production of antisera.

Polyclonal antiserum JB1 against formalinized cells. Confluent growth from five (100 x 15 mm) plates of *C. pylori* 5924 was resuspended in 15 mL of 37% (v/v) formaldehyde and stored at 4 C overnight. The formalinized cells were washed twice in PBS pH 7.4, resuspended in an equal volume of Freund's complete adjuvant (Gibco), and then pipetted vigorously until a creamy, white emulsion formed. An adult New Zealand white rabbit was injected subcutaneously with one mL of this suspension and intramuscularly with another mL of this preparation. Booster injections of 1.5 mL were prepared in Freund's incomplete adjuvant (Gibco) and administered in a similar manner on days 22 and 42 after the initial immunization (81). On day 90, the rabbit was bled, and the sera collected and stored at -20 C in 1.5 mL aliquots. Prebleed sera was taken and used as control sera.

Polyclonal antiserum JB2 against SDS-denatured flagellin. 100 µg of Mono Q purified flagellin was electrophoresed on SDS-PAGE (7.5%). A single lane on the gel was stained with Coomassie blue to localize the position of the flagellin protein band. The flagellin band was then excised from the unstained portion of the gel and macerated with a mortar and pestle in 1.0 mL of sterile PBS, pH 7.4. An equivalent volume of Freund's complete adjuvant was added and the mixture was sonicated on ice for 4 intervals of 15 sec each at power level 3 using a Model W-385 sonicator (Heat Systems Inc., Farmingdale, NY). An adult New Zealand white rabbit was injected subcutaneously with 1 mL of this preparation and

intramuscularly with another mL of this preparation. A booster injection containing 10 μg of purified protein was prepared in a similar manner in Freund's incomplete adjuvant and administered on day 32 (81). The rabbit was bled on day 90, and the sera was stored at -20 C in 1.5 mL aliquots. Prebleed sera was taken and used as control sera.

Polyclonal antiserum JB3 against Mono Q purified flagellin. A 100 μg sample of purified flagellin was resuspended in 1.0 mL of sterile PBS, pH 7.4, and mixed with an equal volume of Freund's complete adjuvant. The mixture was sonicated on ice for 4 intervals of 15 sec each at power level 3 on a W-385 Model sonicator (Heat Systems Inc.). An adult New Zealand white rabbit was injected subcutaneously with 1 mL of this preparation and intramuscularly with another mL of this preparation. Booster injections containing 50 μg and 25 μg of purified protein were prepared in a similar manner in Freund's incomplete adjuvant and administered on days 51 and 59 respectively (81). On day 90, the rabbit was bled, and the sera collected and stored at -20 C in 1.5 mL aliquots. Prebleed sera was taken and used as control sera.

Monoclonal antibody production to flagellin. Hybridomas secreting MAbs to the Mono Q purified protein of *C. pylori* 5294 were prepared by the procedure of Pearson et. al. (111). Two BALB/c mice were each injected intraperitoneally with 500 μL of an emulsion containing Freund's complete adjuvant (Gibco) and 100 μg of purified flagellin. Three weeks later, the mice were boosted intraperitoneally with 500 μL of an emulsion containing Freund's incomplete adjuvant (Gibco) and 60 μg of purified flagellin. Two weeks later, an intravenous injection of 300 μL containing 50 μg of flagellin in sterile PBS, pH 7.4 was administered to each

mouse. Three days later, one mouse was sacrificed. The spleen was removed aseptically and the spleen cells were fused with the nonsecreting myeloma cell line SPO/2-Ag 14 using a 35% (w/v) polyethylene glycol solution (Sigma) at 37 C and adjusted to pH 8.0-8.2 with sterile 0.8 N NaOH. The hybridoma cells were then added to 40 mL of 16% (v/v) methylcellulose solution containing hypoxanthine aminopterin thymidine (HAT) (Gibco). 1 mL aliquots of this suspension were dispersed in 100 x 15 mm sterile petri dishes. After 12 days incubation at 37 C in a microaerophilic environment, colonies appeared. Colonies 0.5 mm in diameter were transfered to hypoxanthine thymidine (HT) (Gibco) medium in 96 well plates. To identify positive clones, supernatants were assayed by ELISA using Mono Q purified flagellin protein as the antigen. Phenotypically stable clones were expanded by growth in culture and then doubly cloned by limiting dilution. The class and subclass of each clone was determined by indirect ELISA (American Qualex International, Inc., La Mirada, CA). For production of ascites fluid, 1.5×10^6 cells/mL were injected intraperitoneally into BALB/c mice primed with pristane (2,4,10,14-tetramethyl-pentadecane) (Sigma). Once the abdomens were visibly swollen, the mice were sacrificed and the ascites fluid was collected. The fluid was centrifuged at 200 x g for 10 min at 20 C. Supernatants were aliquoted to 1 mL vials, and stored at -20 C. Hybridomas were stored in 90% fetal bovine serum (Gibco) and 10% dimethyl sulfoxide in liquid N_2.

Immunoblotting. After SDS-PAGE, separated components were transferred from the slab gel to nitrocellulose paper by the methanol-TRIS glycine (25 mM Tris-HCl, pH 8.3, 192 mM glycine, 20% methanol) system described by Towbin et. al. (128). Electroblotting was carried out in a

transblot apparatus model 160/1.6 (Bio-Rad) overnight at 60 volts. After blocking unreacted sites overnight at 4 C in a 0.25% (w/v) solution of gelatin in 10 mM TRIS, pH 7.4 + 0.9% (w/v) NaCl (TBS Buffer), the nitrocellulose paper was agitated at room temperature for 2 hr with polyclonal antiserum diluted 1:5000, or with monoclonal antibody diluted 1:10,000 in a 1% (w/v) solution of gelatin in TBS. After washing in TBS, the nitrocellulose paper was incubated with alkaline phosphatase conjugated goat-anti-rabbit or goat-anti-mouse antibody (Caltag Laboratories, San Francisco, CA) diluted 1:5000 in a 2% (w/v) solution of gelatin in TBS for 1 hr. Again the blot was washed in TBS. The nitrocellulose paper was then reacted with Detection Buffer (66 µL of nitroblue tetrazolium (Sigma) in 70% (v/v) dimethyl formamide and 50 µL of 5-bromo-4-chloro-3-indoyl phosphate in 100% dimethyl formamide was added to 15 mL of 0.1 M TRIS pH 9.5, 0.1 M NaCl and 50 mM $MgCl_2$ pre-warmed to 37 C) until colour development occurred. Colour development was stopped by placing the blot in distilled water.

Indirect enzyme linked immunosorbent assay (ELISA). The ELISA assay was performed essentially by the method of Engvall and Perlmann (27) with modifications by Richardson et. al. (117). Flat bottomed, 96 well polystyrene plates were coated overnight with 5 µg of protein per well in carbonate-bicarbonate buffer at pH 9.6. Unreacted sites were blocked with 3% (w/v) BSA (Sigma), in PBS, pH 7.4 for 1 hr at 37 C on a shaker table. After washing three times in PBS buffer + 0.5% (v/v) Tween 20, rabbit or mouse antiserum diluted in PBS-Tween buffer containing 1% (v/v) BSA was added to the wells and incubated at 37 C for 1 hr on a shaker table. After washing for three intervals at 10 min each, alkaline phosphatase conjugated goat-anti-rabbit or goat-anti-mouse IgG antiserum (Caltag) diluted 1:5000 in

PBS-Tween + 1% (v/v) was added. The plates were incubated for 1 hr at 37 C on a shaker table. After washing again for three intervals at 10 min each, the substrate, p-nitrophenyl phosphate (Sigma) was dissolved in diethanolamine buffer, pH 9.8 (97% diethanolamine (Sigma), 0.2% NaN_3, 0.1% $MgCl_2$ in distilled water) and added to the wells for incubation in the dark at 37 C for 30 min. Colour development was stopped by the addition of 3 M NaOH. The absorbance was read at 405 nm with an EIA model 310 ELISA reader (Biotek Instruments Inc., Highland Park, VT).

When cells were used as antigens, the cells from one agar plate were resuspended in ice cold PBS pH 7.4 + 1.0% (w/v) glucose, washed twice and then adjusted to give an absorbance at 420 nm which corresponded to 1.5 x 10^9 cells/mL. This suspension was diluted 1:200 and 50 µL samples aliquoted to each well of a flexible, round bottom polyvinyl chloride radioimmuno assay (RIA) microplate (T. Pearson, personal communication). To pellet the bacteria, the plates were centrifuged in a Beckman model TJ-6 bench centrifuge for 5 min at 200 x g at 4 C. The supernatant was removed by inverting the plate and the plate incubated overnight at 37 C.

For glutaraldehyde-fixed cells, 100 µL of freshly prepared 0.25% (v/v) glutaraldehyde in PBS, pH 7.4 (50% solution; BDH Chemicals, Toronto, ON) was added to the cell suspension in PBS, pH 7.4 + 1.0% glucose The plates were then incubated at room temperature for 15 min. The plates were again centrifuged for 5 min and the supernatant removed by inverting the plate. Wells were washed twice by gently adding 100 µL of PBS, pH 7.4 to each well and centrifuging for 5 min. The plates were then sealed with parafilm and stored at 4 C overnight before proceeding with the indirect ELISA assay.

Absorption. To remove antibodies to heat stable epitopes, antisera were incubated with boiled cells of the homologous strain (81). Cells from one plate of confluent growth were harvested and boiled for 2 hr at 100 C. The boiled cells were mixed with an equal volume of antiserum and incubated at room temperature for 2 hr with frequent mixing. Boiled cells were removed from the absorbed antiserum by centrifugation at 10,000 x g for 10 min in a microcentrifuge. The supernatant was carefully removed and incubated with freshly boiled cells twice more. The final absorbed antisera was stored at 4 C until use. To remove antibodies to surface exposed epitopes, antisera were incubated with live cells of the homologous strain.

Indirect immunofluorescence (IFAT). *C. pylori* cells grown on solid agar for 72 hr were gently resuspended in 0.05 M phosphate buffer, pH 7.6. 20 µL was smeared on an acid washed glass slide and allowed to air dry. The smears were fixed in 100% methanol for 15 min and again air dried (80). Polyclonal rabbit antiserum diluted 1:50, or MAb diluted 1:500 in PBS, pH 7.4 containing 5% (v/v) fetal bovine serum (Gibco), and 100 µL was applied to each bacterial smear and incubated at room temperature for 30 min in the case of polyclonal antisera, and 1 hr in the case of MAb. Slides were rinsed with PBS, pH 7.4. and then incubated in the dark for 30 min at room temperature with either goat-anti-rabbit or goat anti-mouse fluorescein conjugated antibody (TAGO Inc., Burlingame, CA) diluted 1:80 in PBS containing 5% (v/v) fetal bovine serum (Gibco). After washing the slides in PBS, pH 7.4, and air drying, one drop of mounting solution (0.0625 % (w/v) n-propyl gallactate in PBS mixed with an equal volume of glycerol) was applied to the slides. The slides were examined and photographed

using a 100 x Neofluor objective on a Zeiss MC63/IIIRS microscope (Carl Zeiss Inc., Munich, West Germany).

Transmission electron microscopy. Formvar coated 100 mesh copper grids (Ernst F. Fullam, Inc., Schenectady, NY) were coated with a liquid culture of *C. pylori* cells for 20 sec and air dried. The grids were then negatively stained with 2% (w/v) phosphotungstic acid (Fisher Scientific, Fair Lawn, NJ) (80) in distilled water, pH 7.2 for 12 sec and examined under a Philips (Philips Electronics Inc., Scarborough, ON) transmission electron microscope 300 at 60 kV.

Immuno-electron microscopy. *C. pylori* cells grown for 72 hr were carefully removed from an agar plate with a loop and gently suspended in 0.05 M phosphate buffer, pH 7.6. A 50 µL aliquot was applied to a piece of parafilm and a 100 mesh formvar coated copper grid was floated on the suspension for 3 min. The grid was then floated on 50 µL of 1.0% (w/v) BSA in PBS, pH 7.4 for 5 min. The grid was then transferred to 50 µL of polyclonal rabbit antiserum diluted 1:10 in PBS-BSA and incubated at room temperature for 30 min. After washing on a drop of PBS-BSA, pH 7.4, the grid was floated on a drop of a 1:20 dilution of goat anti-rabbit IgG for 30 min. In the case of MAbs, grids were floated on 50 µL of monoclonal antibody diluted 1:50 in PBS-BSA and incubated at room temperature for 1 h. After washing on a drop of PBS-BSA, pH 7.4, the grid was floated on a drop of a 1:20 dilution of goat anti-mouse IgG1 (Miles Scientific, Etobicoke, ON) for 30 min (R. Garduno, personal communication). At the end of this incubation, grids were washed by floating for 1 min on four 50 µL aliquots of PBS and BSA (37). Each grid was then incubated with 50 µL of Protein-A-colloidal gold (15 nm; Jannsen Pharmaceutica, Beerse, Belgium) diluted 1:20

in PBS-BSA at room temperature for 30 min. Grids were then washed by floating on three successive 50 μL drops of double distilled water for 5 min intervals before air drying overnight. Grids were then stained for 15 sec with 2.0% (w/v) phosphotungstic acid (Fisher Scientific) pH 7.2 and examined under a Philips 300 transmission electron microscope at 60 kV.

RESULTS

Characterization of Strains

The three *C. pylori* strains used in the study, 5294, 5155, and 5442 were isolated from patients in Australia that were suffering from active chronic gastritis. The strains had been stored in Trypticase soy broth plus 15% glycerol at either -70 C, or in liquid nitrogen. At the initiation of the project, the growth requirements of *C. pylori* were poorly defined. The addition of starch, serum, charcoal or hemin were just a few of the factors that were suggested for addition to culture media (14). Attempts to grow the isolates at 37 C on solid medium containing Blood Agar Base No. 2 plus 5% laked blood, with and without campylobacter antibiotics, in a variety of microaerophilic environments were unsuccessful. These results are summarized in Table 1. The medium that provided best growth was a solid chocolate medium containing the antibiotics vancomycin, trimethoprim, and colymicin. Best growth was obtained when an Oxoid *Campylobacter* GasPaks were used to generate the microaerophilic environment. Another medium which provided good growth of *C. pylori* was Lior's liquid broth. It was found that culturing *C. pylori* on solid media was most advantageous for large scale preparations of cells, whereas culturing *C. pylori* in liquid culture was often advantageous for electron microscopic studies.

Microscopically, the bacteria appeared as small, thin, curved gram-negative rods. S and U or bow shaped cells were common. The ultrastructure of *C. pylori* 5294 is shown in Figure 1. This micrograph illustrates two prominent features of *C. pylori*: the curved shape of the rod and the five sheathed flagella that extend from a single pole. The size of the cell is 1.0 μm x 0.3 μm which is consistent with other reports in the

Table 1. Growth conditions for *C. pylori* at 37 C [a]

	MERCK ANAEROBIC GENERATOR	CARBON DIOXIDE INCUBATOR	OXOID ANAEROBIC GASPAK	OXOID CAMPY GASPAK
Blood Agar Base No.2 + Laked Blood with Campy Antibiotics	-	+	+	+
Blood Agar Base No. 2 + Laked Blood without Campy Antibiotics	-	+	+	+
Chocolate Agar with Campy Antibiotics	ND	++	+++	++++
Chocolate Agar without Campy Antibiotics	ND	++	+++	++++
Lior's Liquid Medium	ND	++	++	++

[a] ++++ is excellent growth, +++ is good growth, ++ is moderate, + is poor growth, -is no growth

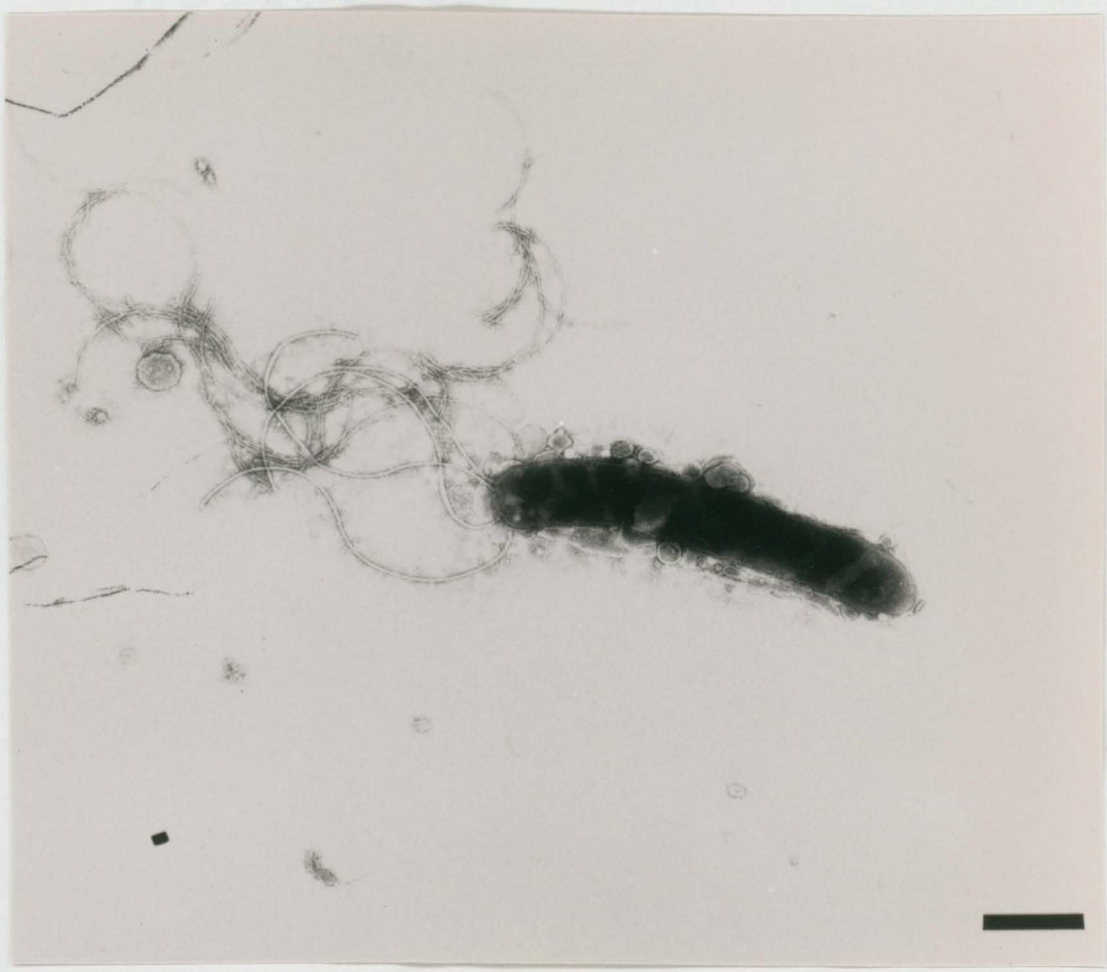


Figure 1. Transmission electron micrograph negatively stained by 2% PTA, pH 7.2 showing the ultrastructure of *C. pylori* 5294. Bar indicates 405 nm.

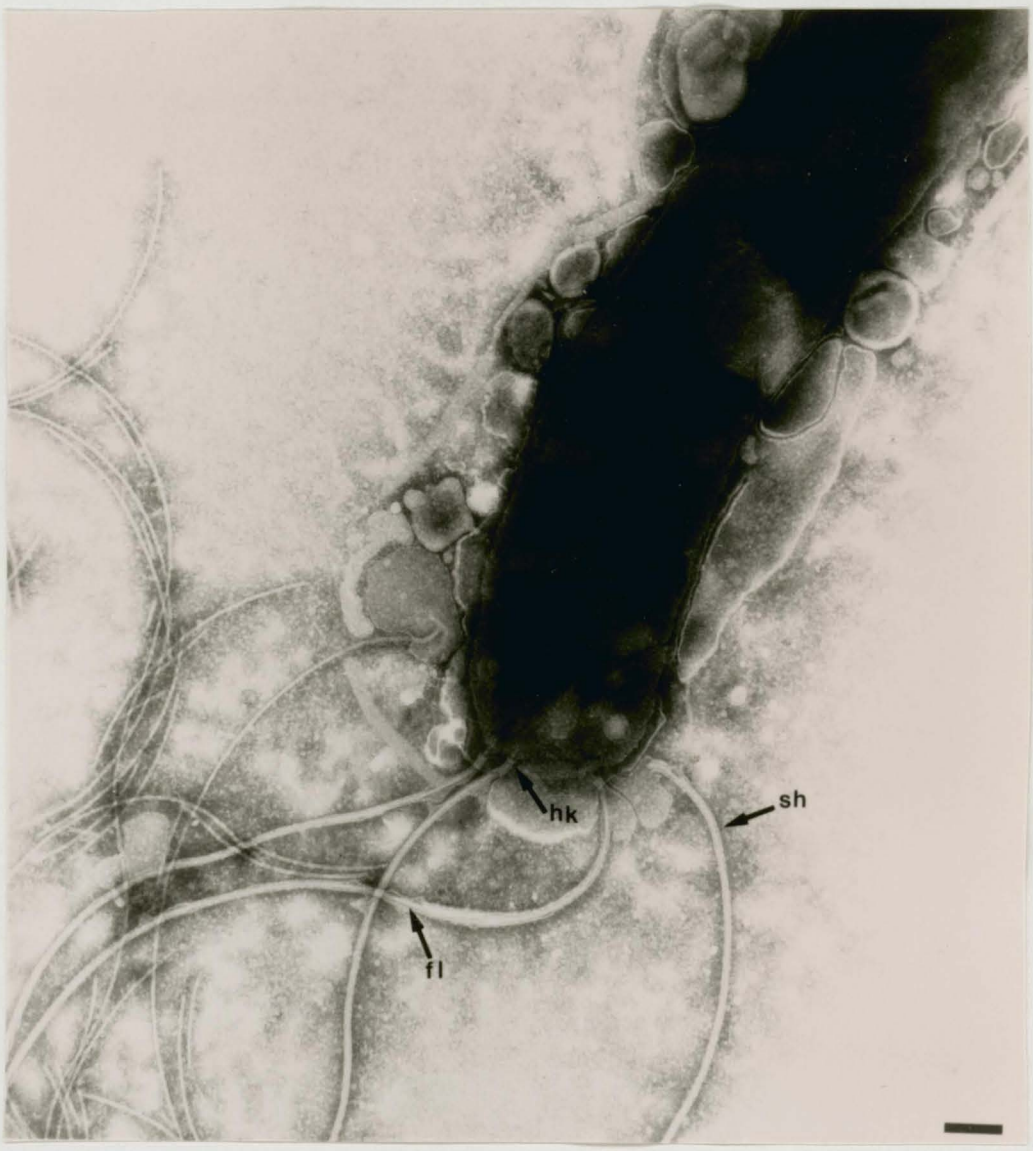


Figure 2. Transmission electron micrograph of the pole of a *C. pylori* 5294 cell negatively stained by 2% PTA, pH 7.2 showing the sheathed flagella, and unsheathed flagellin filaments at higher magnification (fl=flagellin filament; sh=flagellar sheath; hk=flagellar hook). Bar indicates 87 nm.

literature (61). Figure 2 is a higher magnification of the pole showing the sheathed flagellar filaments. The inner flagellar filament has a diameter of 9 nm and the sheathed filament has a diameter of 15 nm. Note the delicate nature of the sheath and how it appears to peel from the filament in some instances, and to separate from the filament in blebs in other cases. Cultures of all three strains were slow growing, and incubation times of 48-72 hr were commonly employed. The colony morphologies of each of the strains studied were identical. They were pinpoint in size, had smooth edges, a convex shape, a glossy surface, a yellow pigment and a gummy consistency.

To confirm the identity of the three strains, the bacteria were biotyped according to the protocol of McNulty and Dent (92). The results of the biochemical characterization of strain 5294 are summarized in Table 2. The other two strains gave identical results. Also shown are the reactions of other species of campylobacters for comparison. Most notable was the strong urease activity of each of the three *C. pylori* strains. This is a hallmark of the species *C. pylori*. The three strains also possessed the enzymes oxidase, catalase, alkaline phosphatase and DNAse, but unlike other *Campylobacter* species, were unable to hydrolyze hippurate or produce hydrogen sulfide. Thus, from the microscopic and macroscopic morphologies, and the results of the biochemical tests, the three strains, 5155, 5442, and 5294 were positively identified as *C. pylori*.

To further compare the similarities among the *C. pylori* strains, the protein profile of each was examined by SDS-PAGE. Figure 3 shows a gel in which whole cell lysates of the *C. pylori* strains were compared with a variety of *Campylobacter* species. As has been reported (112), the protein

Table 2. Biochemical characteristics of C. *pylori* and other *Campylobacter* species [a]

	C. pylori	*C. jejuni*	*C. coli*	*C. fetus*	*C. laridis*	*C. pylori* 5294
Oxidase	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Urease	+	-	-	-	-	+
Alk. phos.	+	-	-	-	-	+
DNAse	+	-	-	-	-	+
Rapid H_2S	-	-	-	-	+	-
Hippurate	-	+	-	-	-	-

[a] + is a positive test reaction, - is a negative test reaction

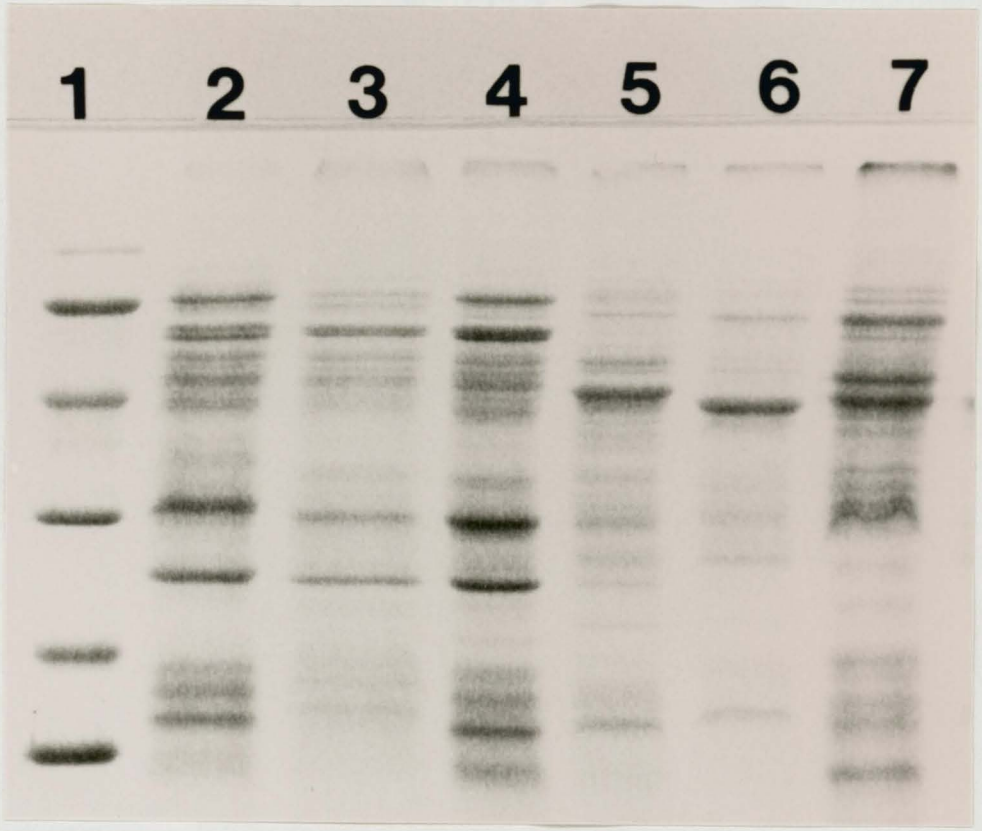


Figure 3. SDS-PAGE of whole cell lysates of *Campylobacter* species stained by Coomassie blue. Lane 1, low Mr standards from top to bottom: 97.4, 66.2, 42.7, 31.0, 21.5, and 14.4 kDa; lane 2, *C. pylori* 5294; lane 3, *C. pylori* 5442; lane 4, *C. pylori* 5155; lane 5, *C. laridis*; lane 6, *C. coli* VC167B; lane 7, *C. fetus* VC78.

profile of the three *C. pylori* strains were similar to each other, but were markedly different from the other campylobacters tested. The SDS-PAGE profiles of *C. pylori* contained seven prominent proteins which corresponding to approximate subunit Mrs (kDa) of 67.0, 60.0, 58.7, 35.0, 27.0, 19.4, and 17.9. Particularly noteworthy in the profile of the *C. pylori* strains was the absence of the single major protein band which corresponds to the porin protein of the true campylobacters.

To complete the initial characterization of *C. pylori* cell structure, the LPS structure of *C. pylori* 5294 was also examined. The outer membrane fraction was isolated, and digested with proteinase K, leaving LPS components intact for staining by silver. The migration pattern was consistent with an LPS containing O-polysaccharide chains of relatively constant chain length (data not shown). By comparison, O-polysaccharide chains are not readily visualized when the technique is applied to other campylobacters, with the exception of *C. fetus* (81).

Isolation and Purification of Flagellin

CsCl Density Gradient Purification. The first method employed to isolate *C. pylori* flagellar filaments was that described by Chandler and Gulasekharam (18). The procedure is based on the fact that the density of intact flagellar filaments in a CsCl gradient is 1.3 g/cm^3. In theory, this property allows the flagellar filaments to form a dense white band in the centrifuge tube that can easily be extracted with a needle and syringe. After shearing, cells were removed by centrifugation, and flagella filaments recovered from the supernatant by filtration. Contaminating nucleic acids and proteins were then degraded by incubation with added ribonuclease and

trypsin, respectively. The success of this protocol relies upon the fact that flagellar filaments are resistant to degradation by trypsin (18). In order to confirm this, a sample of the isolated filaments was analyzed by SDS-PAGE after incubation with the enzymes. As can be seen in Figure 4 all of the proteins contaminating the flagellin were removed by digestion with trypsin. The remaining 58 kDa protein had the same subunit Mr as the protein that cross reacted with antiserum made to the flagellin protein of *C. jejuni* VC74 (72). Shown in Figure 5A is an electron micrograph of the unsheathed flagellar filaments that were contained in the dense white band in the centrifuge tube. This provided the first morphological evidence that the 58 kDa antigenically cross reactive protein of *C. pylori* was flagellin.

One problem encountered with the CsCl purification procedure when applied to the isolation of *C. pylori* flagella was that the trypsin and ribonuclease added during the purification procedure repeatedly contaminated the dense white flagella filament band in the centrifuge tube. In an attempt to solve this problem, it was thought that instead of removing the CsCl salt from the isolated protein by filtration through a membrane with a Mr cut-off of 10,000 daltons, a membrane with a Mr cut-off of 30,000 daltons should be used since the Mr of trypsin is 23.8 kDa and the Mr of ribonuclease is 13.7-14.7 kDa. However, as is evident from the SDS-PAGE gel shown in Figure 6, the modification was not successful in overcoming this problem, and the flagella filaments could not be freed of trypsin contamination, perhaps because of hydrophobic interaction between the filaments and the enzyme. An alternative method of isolating flagellar filaments was therefore sought.

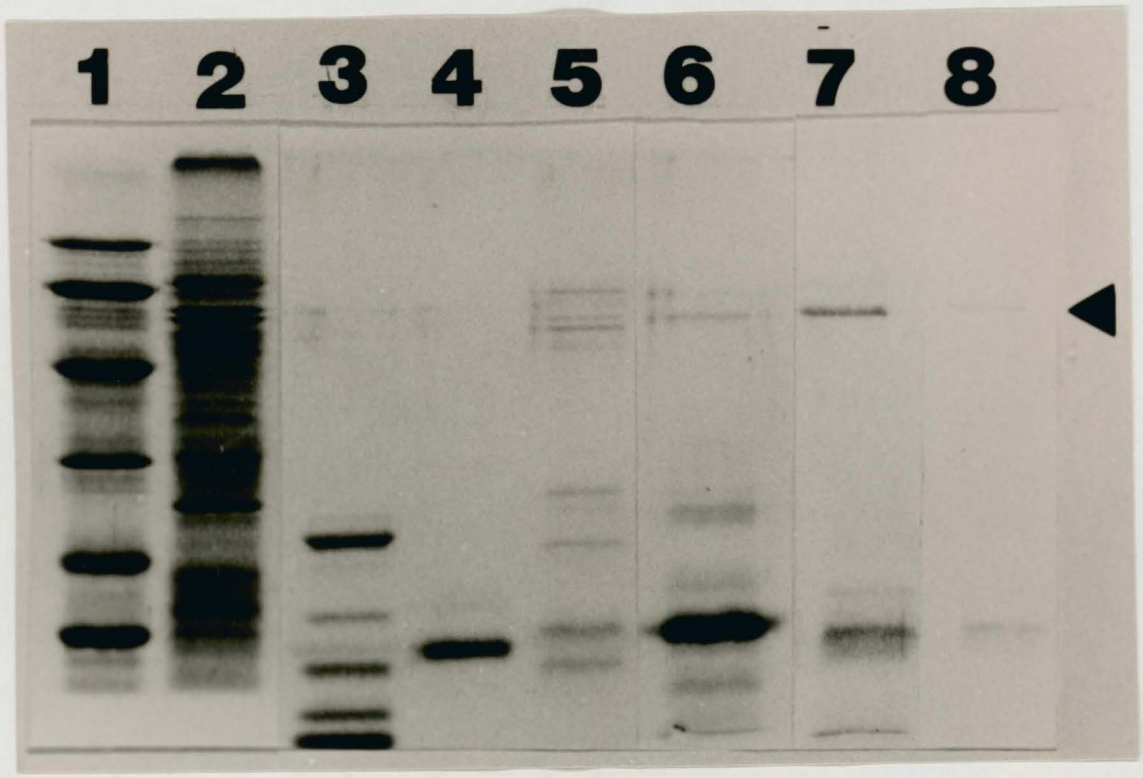


Figure 4. SDS-PAGE analysis of the CsCl density gradient purification of C. pylori 5294 flagellar filaments stained by Coomasie blue. Lane 1, low Mr standards, from top to bottom: 97.4, 66.2, 42.7, 31.0, 21.5, 14.4 kDa, Lane 2, whole cell lysate; Lane 3, trypsin (1 mg/ml), 10 µl; Lane 4, ribonuclease (0.5 mg/ml) 10 µl; Lane 5, sheared flagellar filaments; Lane 6, sheared flagellar filaments after incubation with ribonuclease and trypsin; Lane 7, band obtained from CsCl density gradient ultracentrifugation procedure, after removal of CsCl by Amicon PM10 membrane filtration; Lane 8, band obtained from CsCl density gradient ultracentrifugation procedure, after removal of CsCl by Amicon YM30 membrane filtration. Arrow indicates 58 kDa flagellin subunit.

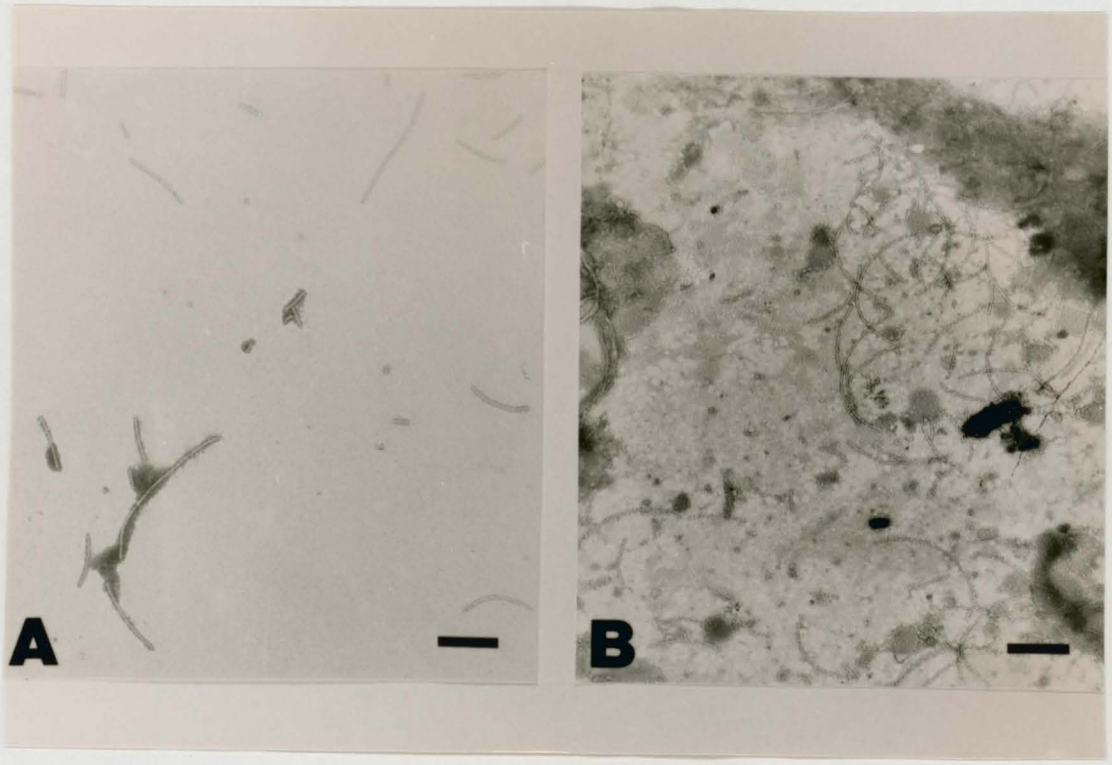


Figure 5. Transmission electron micrographs of flagellin filaments isolated from *C. pylori* cells by two different methods, negatively stained by 2% PTA, pH 7.2. **A**. Flagella filaments remaining on the Amicon PM10 membrane in the CsCl density gradient purification procedure. **B**. Flagellin filaments and contaminating material in 100,000 x g pellet obtained after shearing of cells during the pH 2 acid disassociation-differential ultracentrifugation procedure. Bar indicates 144 nm.

pH 2.0 disassociation and differential ultracentrifugation. The method of Logan and Trust (81) had previously been successfully employed to isolate flagellin from the non-sheathed flagella of enteropathogenic campylobacters. Unlike the CsCl isolation procedure in which the final product isolated was fragmented flagella filament, the final product isolated in the method of Logan and Trust (81) is a mixture of flagellin monomers, as well as reassociated pieces of flagella filament. In this procedure sheared flagella are first collected by ultracentrifugation. Shown in Figure 5B is an electron micrograph of intact unsheathed flagellar filaments present in this 100,000 x g pellet fraction to illustrate the extent of contamination by non-flagella material. Depolymerization of the flagellar filaments into their constituent *C. pylori* flagellin monomers is then brought about by addition of HCl to lower the pH from 7.0 to 2.0. At pH 2.0, contaminating membrane material together with insoluble proteins released by shearing of the cells and initial ultracentrifugation, are pelleted by the second round of ultracentrifugation. The supernatant which contains flagellin monomers is then neutralized with NaOH. In response to this change in pH, some flagellin monomers repolymerize into flagella filaments. This was evident from the formation of a fine white precipitate upon neutralization. Shown in Figure 6 is the SDS-PAGE profile of the isolated *C. pylori* flagellar filaments before pH 2.0 disassociation, after pH disassociation, and of the white precipitate that formed upon the addition of NaOH. A major protein in all three samples had a subunit Mr of approximately 58 kDa. This subunit Mr was consistent with the subunit Mr of flagellin isolated using the CsCl gradient procedure. However the 58 kDa protein isolated using the pH 2.0 disassociation-differential ultracentrifugation procedure was clearly

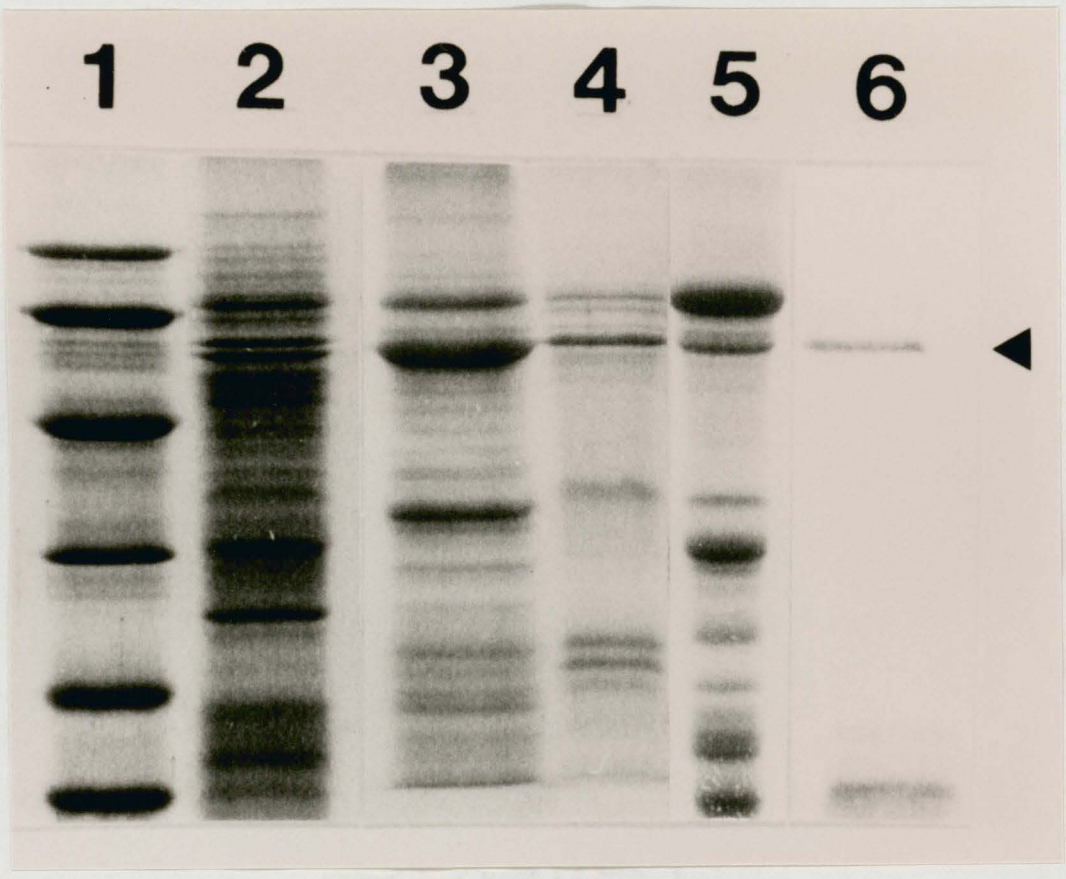


Figure 6. SDS-PAGE analysis of pH 2.0 acid disassociation-differential ultracentrifugation isolation of flagellar filaments from *C. pylori* 5294 stained by Coomassie blue. Lane 1, low Mr standards, from top to bottom: 97.4, 66.2, 42.7, 31.0, 21.5, 14.4 kDa; Lane 2, whole cell lysates; Lane 3, 100,000 x g pellet before pH 2.0 disassociation; lane 4, preparation after pH 2.0 disassociation; Lane 5, white precipitate that formed upon neutralization with NaOH; Lane 6, 58 kDa protein in band obtained from CsCl density gradient ultracentrifugation procedure.

in all three samples had a subunit Mr of approximately 58 kDa. This subunit Mr was consistent with the subunit Mr of flagellin isolated using the CsCl gradient procedure. However the 58 kDa protein isolated using the pH 2.0 disassociation-differential ultracentrifugation procedure was clearly contaminated with other proteins including urease. In fact the major protein band seen in Figure 6 lane 5 is the Mr 62 kDa subunit of the urease complex. Because the various contaminating proteins covered a wide range of subunit Mr's, gel permeation chromatography was then tested to separate the 58 kDa flagellin subunit from these other proteins.

Gel Filtration Chromatography. Concentrated, neutralized supernatant from the pH 2.0 disassociation-differential ultracentrifugation procedure was loaded onto a Superose 12 column (Pharmacia) using FPLC.. The resulting chromatogram using this technique, which separates proteins on the basis of Mr, is shown in Figure 7. Inset is the SDS-PAGE analysis and immunoblot analysis with anti-*C. jejuni* flagellin antiserum SML2. The 58 kDa flagellin was clearly contained in the major peak eluted from the column, and the fraction contained only traces of contaminating proteins.

Ion exchange chromatography. In an effort to obtain even greater purification, anion exchange chromatography was also performed. Concentrated, neutralized supernatant from the pH 2.0 disassociation-differential ultracentrifugation procedure was loaded onto a Mono Q (Pharmacia) anion exchange column, and proteins were eluted by NaCl. The chromatogram for this separation procedure is shown in Figure 8. The arrowed fraction that eluted from the column containing the purified 58 kDa flagellin subunit was analyzed by SDS-PAGE and immunoblotting with anti-*C. jejuni* flagellin antiserum SML2.

Figure 7. Purification of *C. pylori* 5294 flagellin by Superose 12 gel filtration. Chromatogram of pH 2.0 solubilized 100,000 x g pellet injected onto the Superose column. **A**. SDS-PAGE stained by Coomassie blue. Lane 1, fraction collected under arrowed peak showing partially purified *C. pylori* 58 kDa flagellin; Lane 2, pH 2.0 solubilized 100,000 x g pellet injected onto the Superose column; Lane 3, low Mr standards, from top to bottom, 97.4, 66.2 42.7, 31.0, 21.5, 14.4 kDa. **B**. Immunoblot with a 1:500 dilution of antiserum SML2 against *C. jejuni* VC74 flagellin.

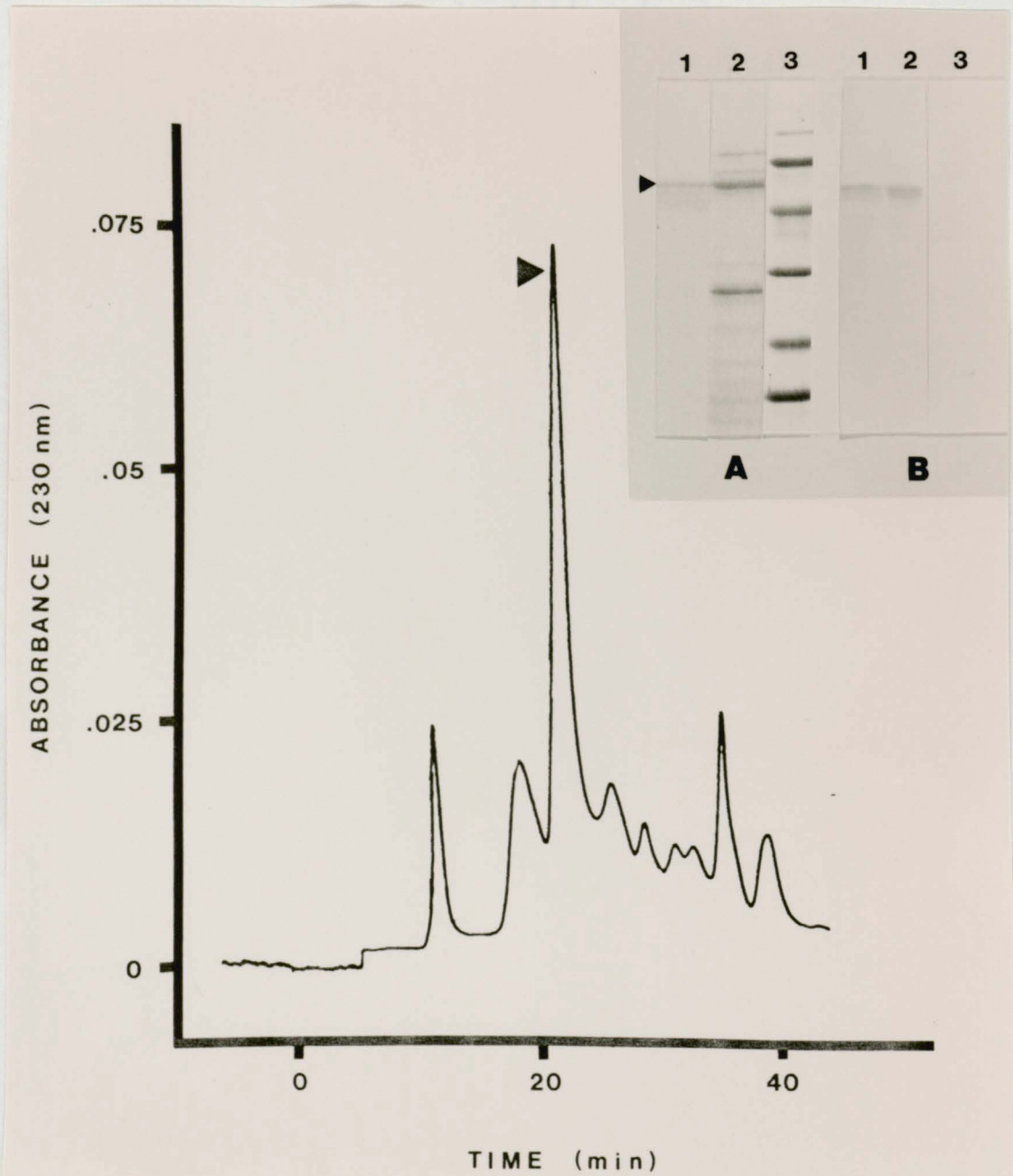
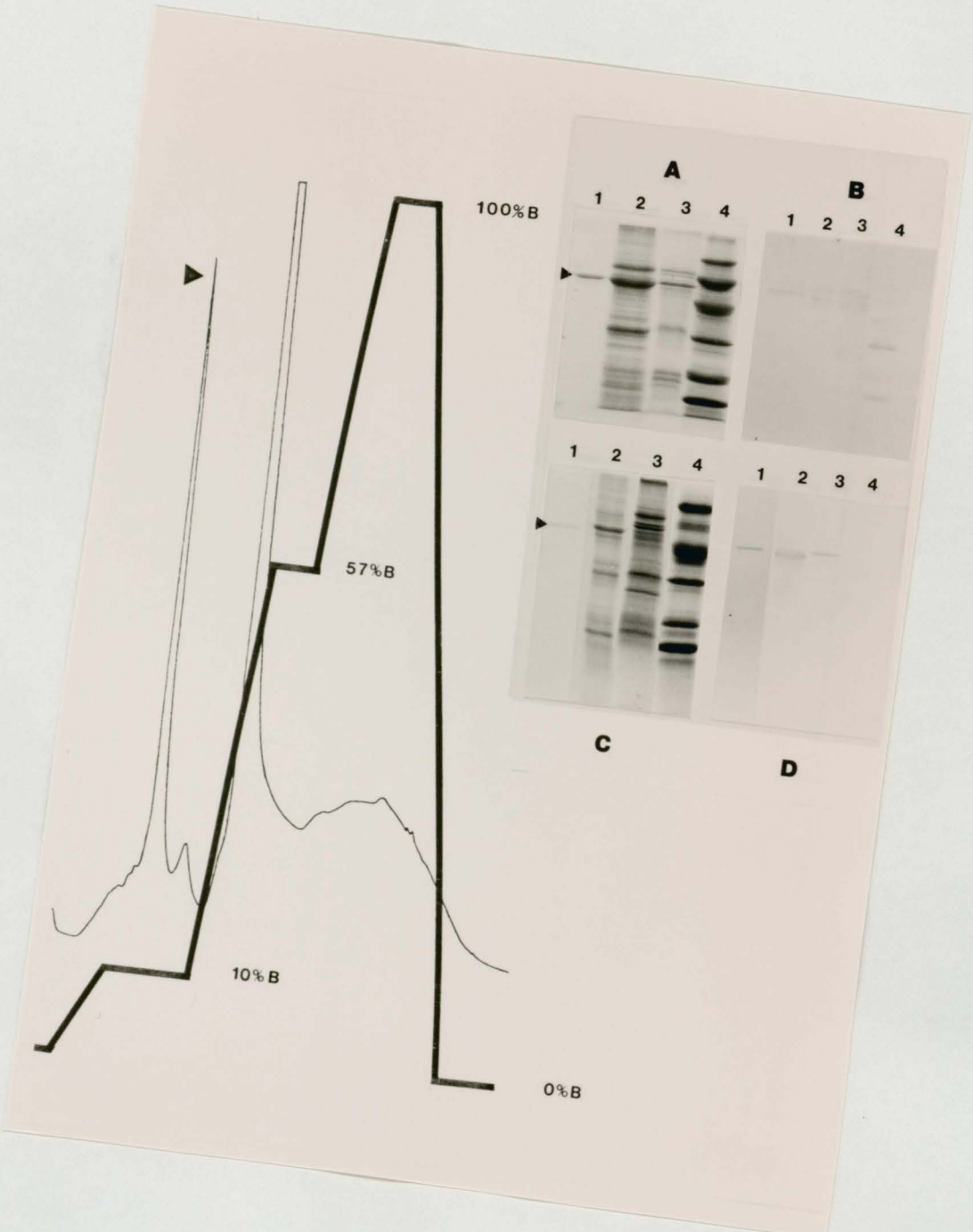

1 2 3 1 2 3
A
B
.075
.05
.025
0
ABSORBANCE (230 nm)
0
20
40
TIME (min)

Figure 8. Purification of *C. pylori* 5294 flagellin by FPLC using Mono Q anion exchange chromatography. Chromatogram of pH 2.0 solubilized 100,000 x g pellet injected onto a Mono Q column at pH 6.2. **A** and **C** . SDS-PAGE stained by Coomassie blue **B**. Immunoblot with 1:500 dilution of antiserum AL1 directed toward whole cells of *C. pylori* 5155. **D.** Immunoblot with 1:500 dilution of antiserum SML2 directed against *C. jejuni* VC74 . Lane 1, fraction collected under arrowed peak showing partially purified *C. pylori* 58 kDa flagellin; Lane 2, pH 2.0 solubilized 100,000 x g pellet injected onto the Mono Q column; Lane 3,panel A, 100,000 x g pellet before pH 2.0 disassociation;Lane 3, panel C, whole cell lysate from *C. pylori* 5294 Lane 4, low Mr standards, from top to bottom: 97.4, 66.2, 42.7, 31.0, 21.5, 14.4 kDa.


100%B
57%B
10%B
0%B
A
B
C
D
1 2 3 4
1 2 3 4
1 2 3 4
1 2 3 4

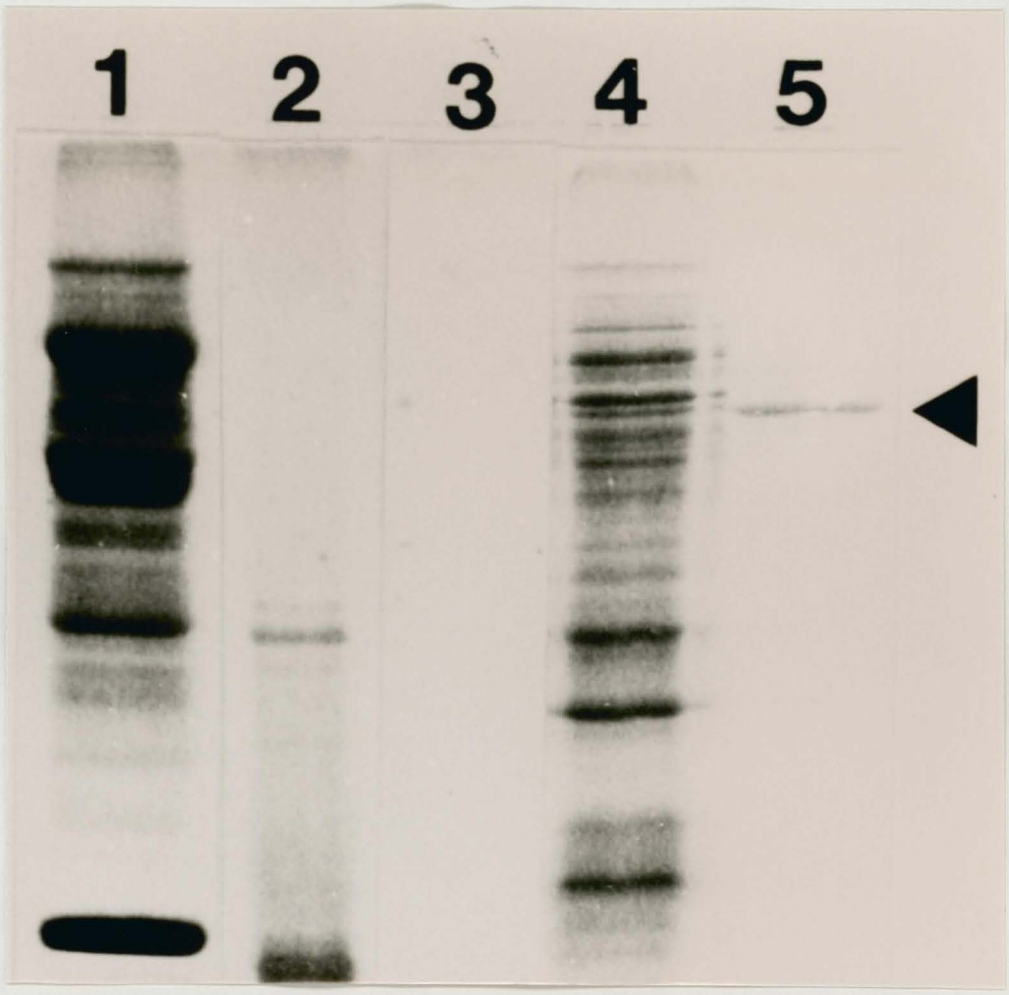


Figure 9. SDS-PAGE analysis of Mono Q purified *C. pylori* 5294 flagellin for presence of LPS stained by silver. Lane 1, low Mr standards, from top to bottom: 97.4, 66.2, 42.7, 31.0, 21.5, 14.4 kDa; Lane 2, 100,000 x g pellet before pH 2.0 disassociation after digestion with proteinase K; Lane 3, 58 kDa flagellin-containing peak a from the Mono Q column after digestion with proteinase K; Lane 4, 100,000 x g pellet before pH 2.0 disassociation prior to treatment with proteinase K stained by Coomassie blue; Lane 5, 58 kDa flagellin-containing peak a from the Mono Q column prior to treatment with proteinase K stained by Coomassie blue. LPS appears to be absent from the proteinase K digested sample of the arrowed peak in Figure 8.

Interestingly, the protein was also reactive to antiserum AL1 produced to whole cells of *C. pylori* strain 5155 indicating the presence of cross-reactive epitopes among *C. pylori* flagellins (inset). Antiserum AL1 had been produced before the initiation of this project, and unfortunately the low titer of the antiserum provided blot reactions of low colour intensity. To confirm that the purified 58 kDa protein was not contaminated with LPS, the fraction collected in the arrowed peak was incubated with proteinase K and stained for the presence of LPS with silver. No LPS was detected (Figure 9, lane 3).

Because the 58 kDa flagellin could be successfully purified by simple FPLC using a Mono Q (Pharmacia) anion exchange column, a multiple step purification system was not pursued, and the anion exchange procedure was used for flagellin purification for the reminder of the study.

Biochemical Characterization of Flagellin

Isoelectric Point Determination. The isoelectric point was determined by isoelectric focussing in a mini-gel system under non-denaturing conditions. The protein focused into a single band at a pI equal to 6.0 (Fig. 10) showing that in its non-denatured conformation the 58 kDa protein of *C. pylori* 5294 was an acidic protein.

N-Terminal Amino Acid Sequence Analysis. The N-terminal amino acid sequence of the *C. pylori* 5294 flagellin was determined for the first 24 residues (Table 3). This sequence contained 50% hydrophobic amino acids, including a methionine at residue 11, as well as 33% neutral acidic amino acids. None of the first 24 residues were charged. Table 3 also shows the alignment of the N-terminal sequences of the flagellins of *C. pylori* 5294, *C. coli* VC167 (79), *C. jejuni* VC91 (79), *S. typhimurium*, (60) and *B. subtilis* (20).

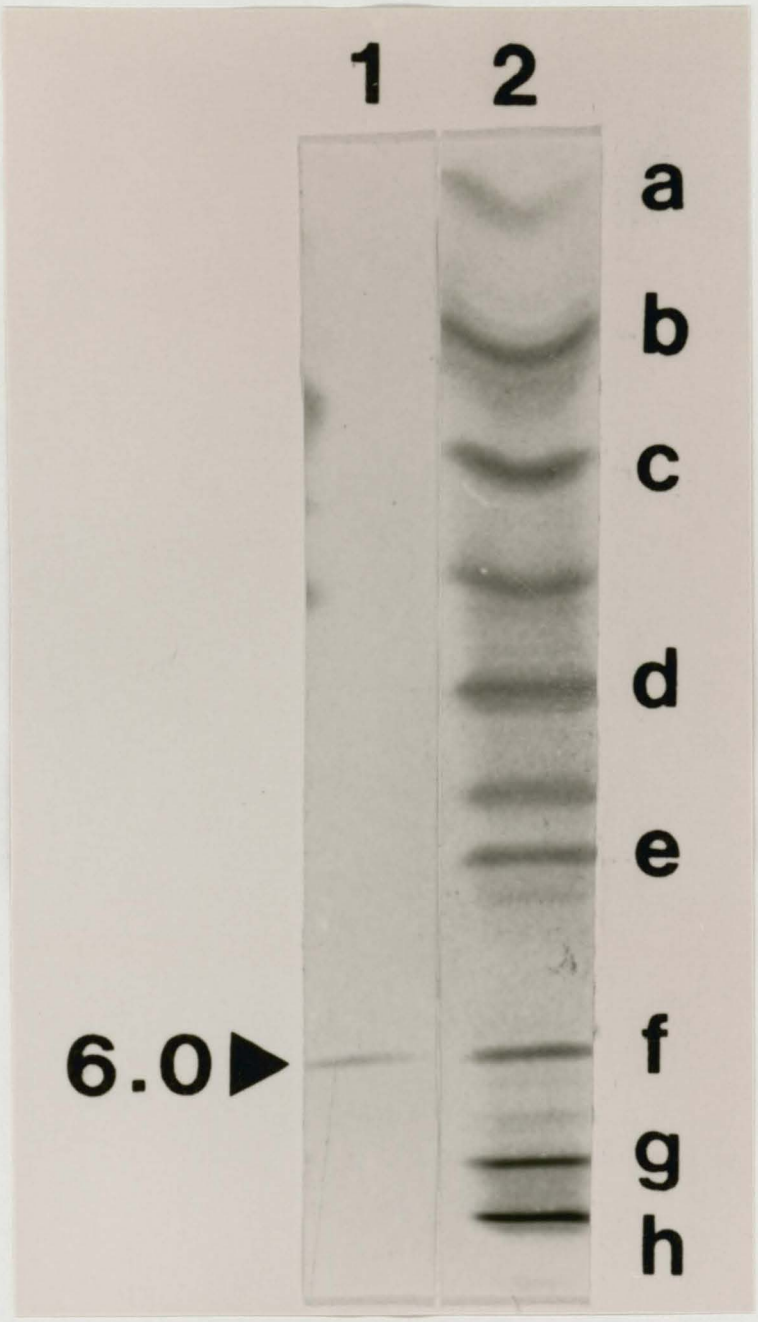


Figure 10. Isoelectric focusing gel of purified *C. pylori* 5294 flagellin stained by Coomassie blue. Lane 1, 58 kDa flagellin, arrow indicates pI of 6.0. Lane 2, Bio-Rad pI standards in the pH range 3-10, labelled as **a**. 9.60, **b**. 8.80, **c**. 8.05, **d**. 7.0, **e**. 6.5, **f**. 6.0, **g**. 5.10, **h**. 4.65.

TABLE 3. N-terminal amino acid sequence of *C. pylori* 5294 flagellin, and other flagellins

Organism	Residue [a]		
	10	20	
C. pylori 5294	AFQVNTNINA	MNAGVQSALT	QNAL
C. coli VC167, P1 [b]	G·RI···VA·	L··KAN·D·N	SRS·
C. coli VC167, P2 [b]	G·RI···VA·	L··KAN·D·N	SRS·
C. jejuni VC91 [c]	G·RI···VA·	L··KAN·D·N	SKS·
S. typhimurium [d]	·QVI···SLS	LLTQNNLNKS	·S··
B. subtilis [e]	RI·H·IA·	L·TLNRLSSN	NS·S

[a] Amino acid residues are designated by the single letter nomenclature. ·, is residue homologous with *C. pylori* 5294 sequence, conservative changes are underlined.

[b] P1 is antigenic phase 1 flagellin, P2 is antigenic phase 2 flagellin (79).

[c] Sequence obtained from purified protein (79)

[d] Sequence derived from DNA sequence of *H-1*^{i} gene (60)

[e] Sequence derived from DNA sequence (20)

TABLE 4. Amino acid composition of *C. pylori* 5294 flagellin, and other flagellins

Amino acid	No. of residues / flagellin subunit								
	C. coli VC167		*C. jejuni* [a]				*S.typhi-murium* [d]	*B.subt-ilis* 168 [e]	*C. pylori* 5294
	P1[b]	P2[b]	VC74	VC91	VC185	81116 [c]			
Asx	86	81	85	91	82	90	79	49	74
Thr	40	38	43	47	46	55	55	18	35
Ser	73	68	70	74	70	71	40	24	54
Glx	53	53	65	60	50	48	47	42	48
Pro	0	0	0	0	0	0	4	2	1
Gly	68	69	101	79	91	71	39	19	63
Ala	72	68	66	82	74	68	61	40	70
Val	29	29	32	29	32	34	31	14	47
Met	14	14	11	13	11	11	3	8	5
Ile	50	48	35	37	49	48	27	24	39
Leu	37	36	40	41	48	39	40	29	45
Tyr	8	8	10	8	10	9	13	1	9
Phe	19	18	18	15	16	19	6	5	12
His	4	4	2	0	1	1	1	4	3
Lys	27	27	34	32	41	33	29	16	31
Arg	17	17	18	16	12	16	14	15	25
Cys	0	0	0	0	0	0	0	0	0
Trp	0	0	0	0	0	0	0	0	0
Residues/mol	598	578	630	624	633	613	489	310	560
Mr apparent (x 1,000)	61.5	59.5	63.8	63.2	63.8	62.9	51.2	33.3	58
Hydrophobic residues (%) (V, M, I, L, A, F, W, P)	37	37	33	35	36	36	35	39	39

[a] Data from Logan et. al. (79). [b] P1 is antigenic phase 1 flagellin, P2 is antigenic phase 2 flagellin (79).[c] Data from Newell et al. (107). [d] Data from Joys (60). The data was calculated based on the DNA sequence of the *H-1 i* gene (60) [e] Data from Delange et al. (20).

Residues identical to those in the *C. pylori* sequence are indicated by a dot, and conservative changes are underlined.

Amino Acid Composition Analysis. Amino acid composition analysis of the flagellin purified from *C. pylori* 5294 showed that the protein contained approximately 560 residues per molecule (Table 4), with a predicted Mr of 58,000 in keeping with the 58 kDa estimated by SDS-PAGE. Asx, ala, gly, ser, glx, val, leu and ile were prominent. Particularly significant was the absence of cysteine and trace levels of tryptophan residues. It is a characteristic of flagellin that the protein lacks both these residues. To confirm their absence, potential cysteine residues were pyridylethylated and potential tryptophan residues were protected from oxidation with thioglycollic acid, and the determinations repeated. Again no cysteine or tryptophan residues were detected. Also characteristic of flagellins was the low pro content. The calculated relative hydrophobicity (assuming val, met, ile, leu, ala, phe, trp and pro) was approximately 39%. Table 4 shows that the amino acid composition of the *C. pylori* 58 kDa protein was consistent with compositions reported for the flagellins of *C. jejuni* (79, 107) and *C. coli* (79), *S. typhimurium* (60), and *B. subtilis* (20).

Immunochemical Characterization of Flagellin

To further examine the antigenic structure of *C. pylori* flagellin, and to provide information on the relatedness of the *C. pylori* protein to the flagellins of other bacteria, polyclonal and monoclonal antibodies were produced to *C. pylori* flagellin. Three different polyclonal antisera were raised in rabbits. Antiserum JB1 was raised to formalinized whole cells to determine whether flagellin was immunogenic when animals were exposed to sheathed intact flagella. This antiserum was also used to

examine for the presence of flagellin in the flagella sheath. Antiserum JB2 was produced to SDS-linearized purified flagellin monomers, and antiserum JB3 was raised against Mono Q purified flagellin to ensure that both linear and topographically assembled epitopes would be represented in the immunological analysis. Hybridomas secreting monoclonal antibodies to *C. pylori* flagellin were obtained according to the method of Pearson et al. (111) by immunizing mice with Mono Q purified flagellin.

Immunological Characterization of Flagellin

Polyclonal Antibody Analysis.

Immunoblot and ELISA analysis. The 58 kDa flagellin was a good immunogen when rabbits were immunized with SDS-linearized purified flagellin monomers, with Mono Q purified flagellin, and with intact formalinized *C.pylori* cells. For example, all three antisera were reactive in immunoblot assays at a dilution of 1:5,000 (Figure 11). ELISA quantitation indicated that Mono Q purified flagellin was the best immunogen, providing antiserum with both a higher titer and a stronger absorbance signal than antiserum prepared against SDS-linearized flagellin (data not shown). This was so when either purified flagellin or flagella filaments were used as the antigen. This likely reflects the presence of both topographically assembled epitopes and linear epitopes on the Mono Q purified protein which was used as the immunogen, and the absence of topographically assembled epitopes on SDS-linearized flagellin. Antiserum JB1 displayed reactivity which was virtually mid-point between the reactivities of antisera JB2 and JB3 as is shown in Figure 12.

Immunoblot analysis with both of the polyclonal antisera to purified *C. pylori* 5294 flagellin showed the presence of cross-reactive epitopes on the

Figure 11 Immunoblot analysis of 58 kDa flagellin protein of *C. pylori* 5294 at various stages in purification with polyclonal antisera JB1, JB2, and JB3 diluted 1:5000. **A**. Lanes 1-4, SDS-PAGE; Lanes 5-8, Immunoblot with antiserum JB2. Lane 1, 58 kDa Mono Q purified flagellin, Lane 2, pre pH 2.0 preparation of flagellar filaments; Lane 3, post pH 2.0 preparation of flagellar filaments; Lane 4, low Mr standards from top to bottom: 97.4, 66.2, 42.7, 31.0, 21.5, 14.4 kDa. **B**. Lanes 1-4, SDS-PAGE; Lane 5, Immunoblot with antiserum JB1; Lanes 6-8, Immunoblot with antiserum JB3. Lanes 1 and 2, Mono Q purified 58 kDa flagellin, Lane 3, post pH 2.0 preparation of flagellar filaments; Lane 4, low Mr standards. **C**. Lane 1, SDS-PAGE of CsCl isolated flagellar filaments (contaminating band at 23.8 kDa is trypsin); Lane 2, Immunoblot with antiserum JB2. SDS-PAGE stained by Coomassie blue.


1 2 3 4 5 6 7 8
A
1 2 3 4 5 6 7 8
B
1 2
C

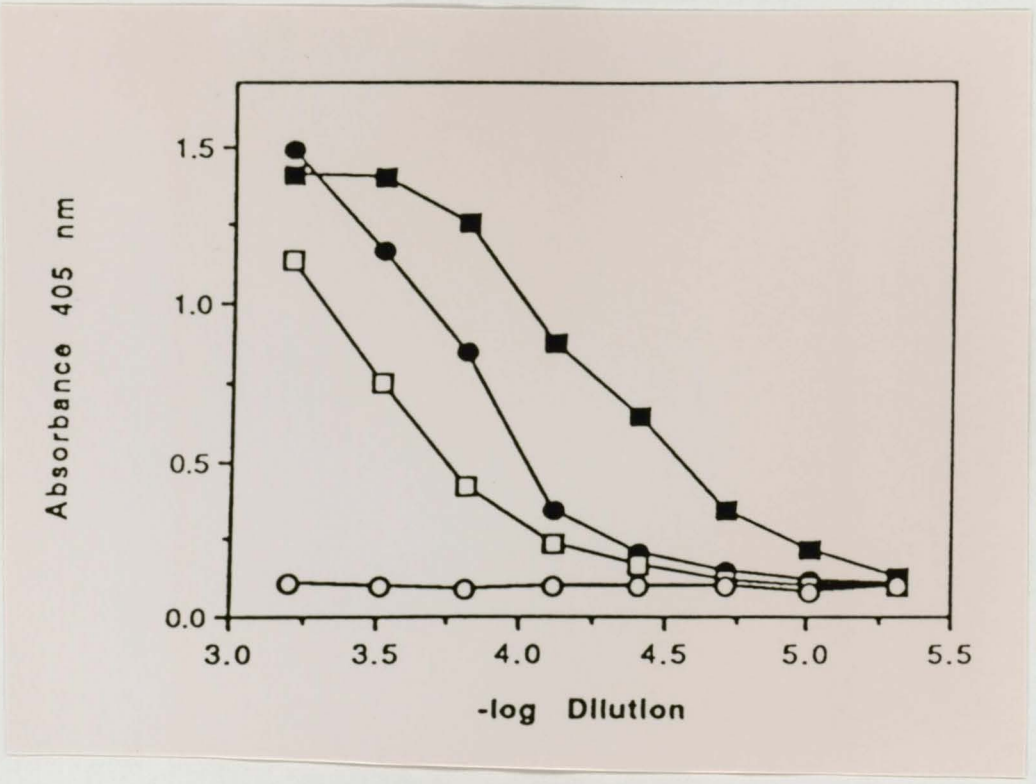


Figure 12. ELISA of antisera JB1, JB2 and JB3 with the 58 kDa flagellin of *C. pylori* 5294: (●) Antiserum JB1; (□) Antiserum JB2; (■) Antiserum JB3; (○) Normal Rabbit Serum.

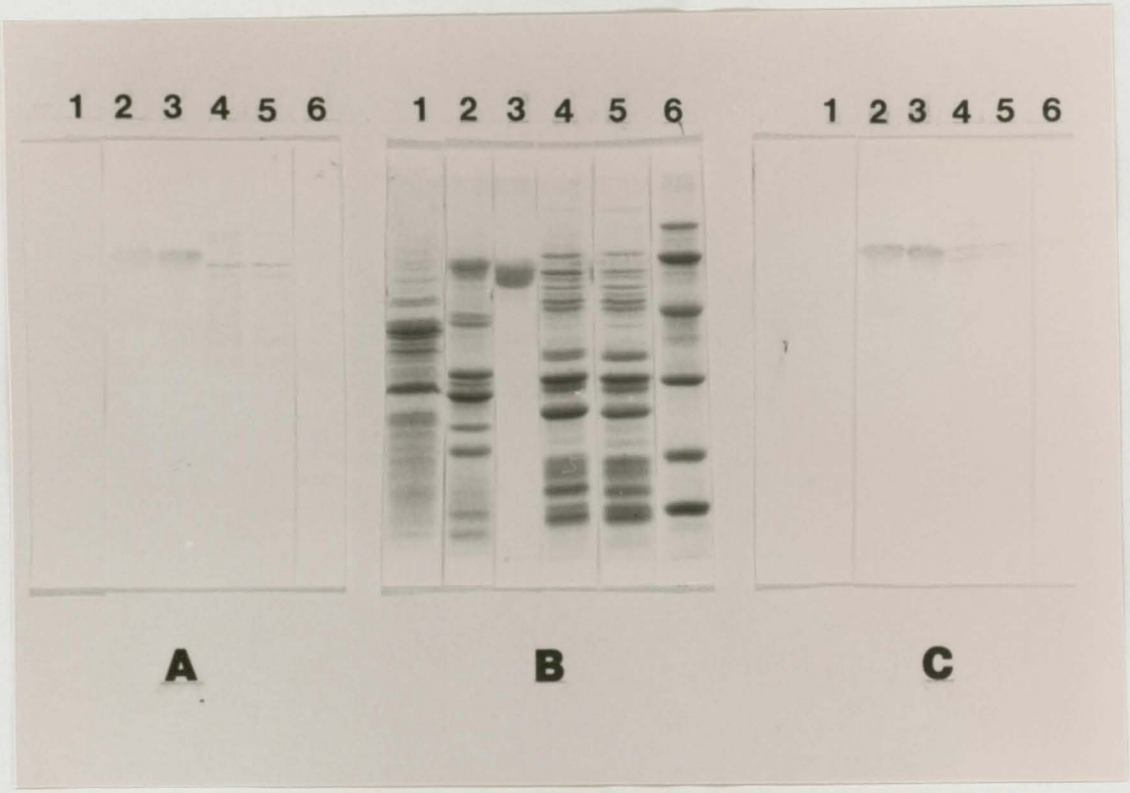


Figure 13. Immunoblot analysis of cross reactivity of flagellin epitopes with polyclonal antisera JB2 and JB3 diluted 1/5000. **A**. Immunoblot with antiserum JB2. **B**. SDS-PAGE stained by Coomassie blue. **C**. Immunoblot with antiserum JB3 . Lane 1, glycine extract of *S. typhimurium*; Lane 2, glycine extract of *C. jejuni* VC74; Lane 3, purified *C. coli* VC167B flagellin; Lane 4, glycine extract from *C. pylori* 5442; Lane 5, glycine extract from *C. pylori* 5155; Lane 6, low Mr standards, from top to bottom: 97.4, 66.2, 42.7, 31.0, 21.5, 14.4 kDa.

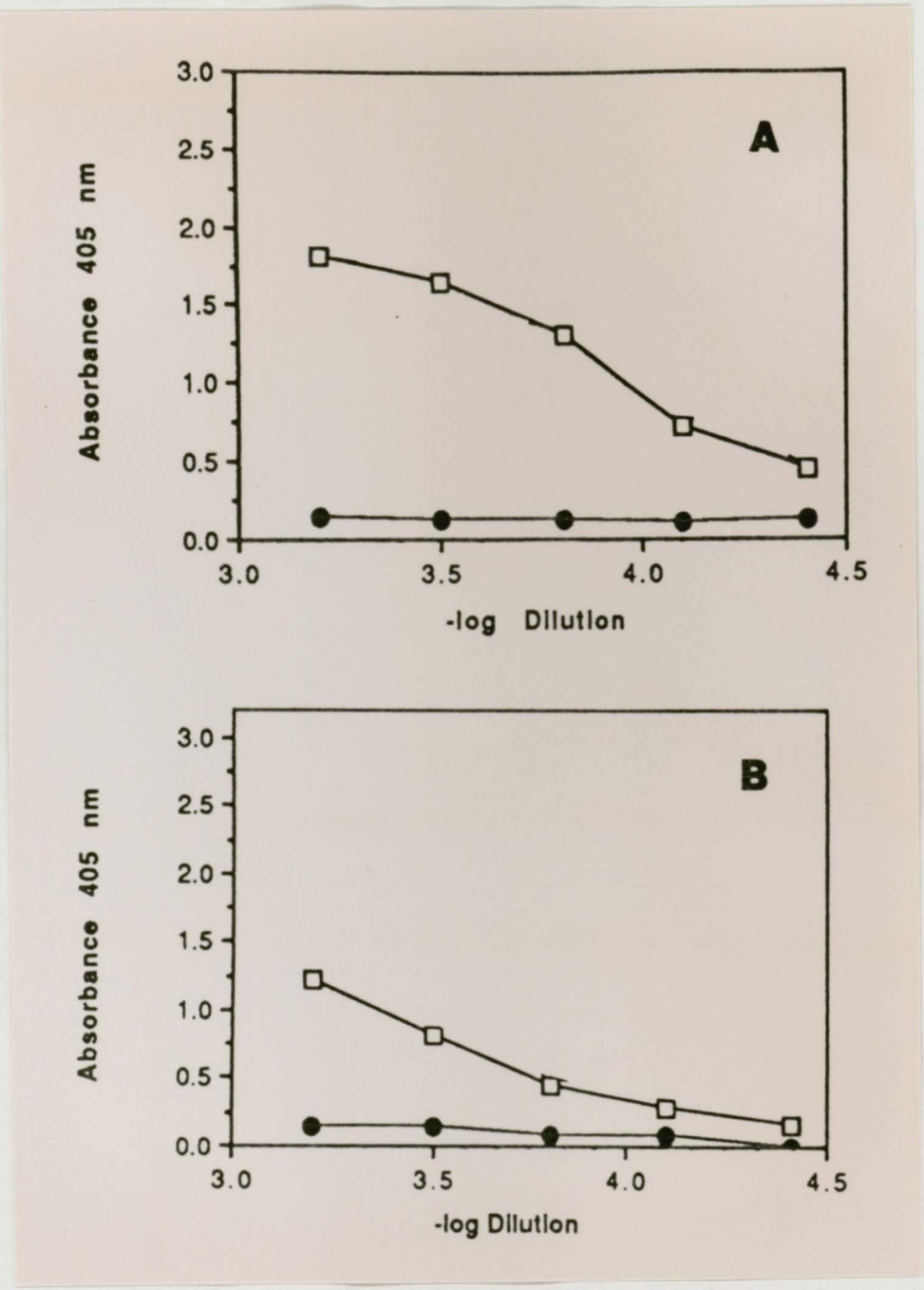


Figure 14 ELISA of polyclonal antiserum JB2 with purified flagellin from **A**. *C. jejuni* VC74 (□). **B**. *C. coli* VC167B (□). Normal Rabbit Serum (●).

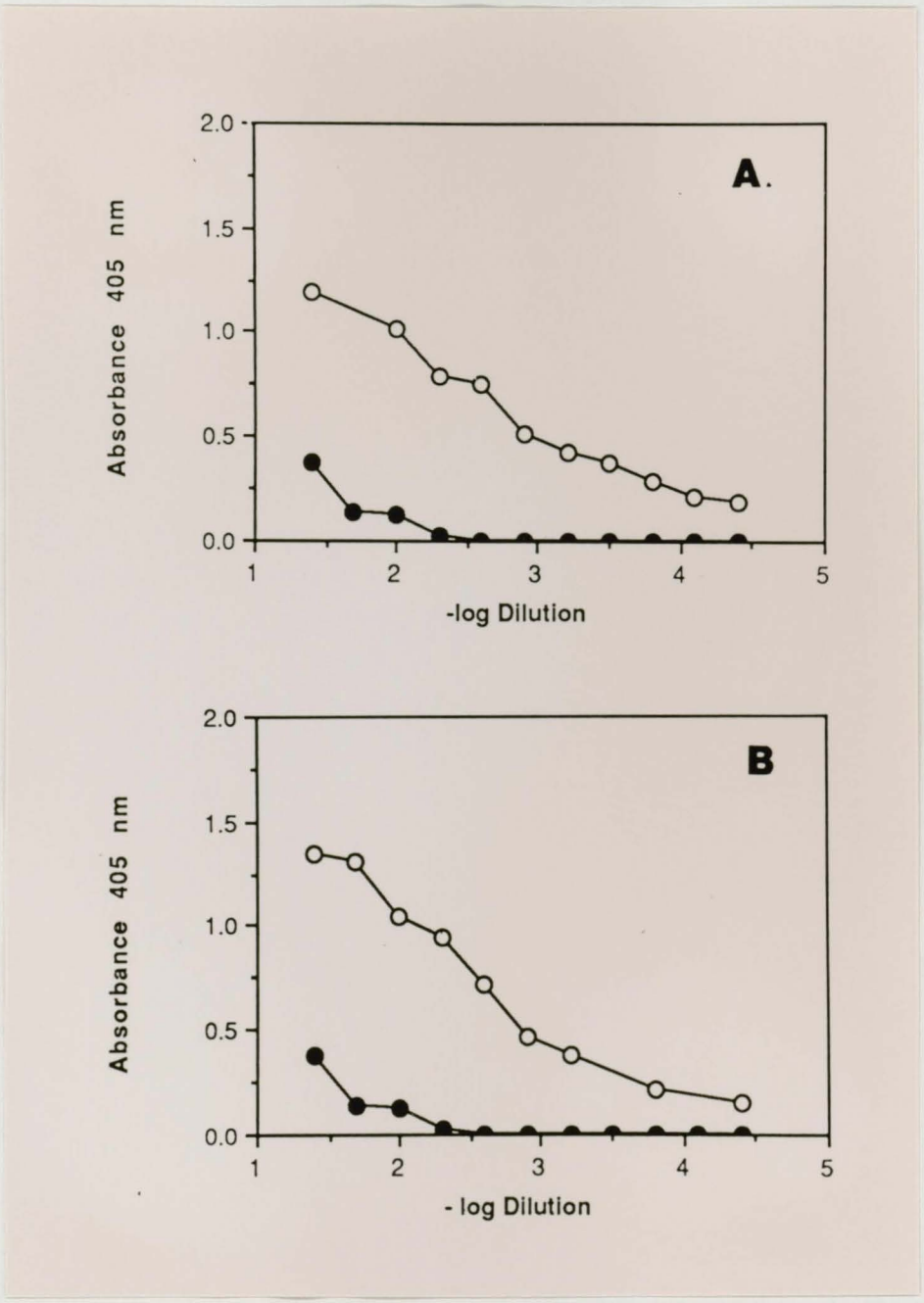


Figure 15. ELISA of polyclonal antiserum JB2 with whole cells of **A**. *C. pylori* 5155 (○). **B**. *C. pylori* 5442 (○). Normal Rabbit Serum (●).

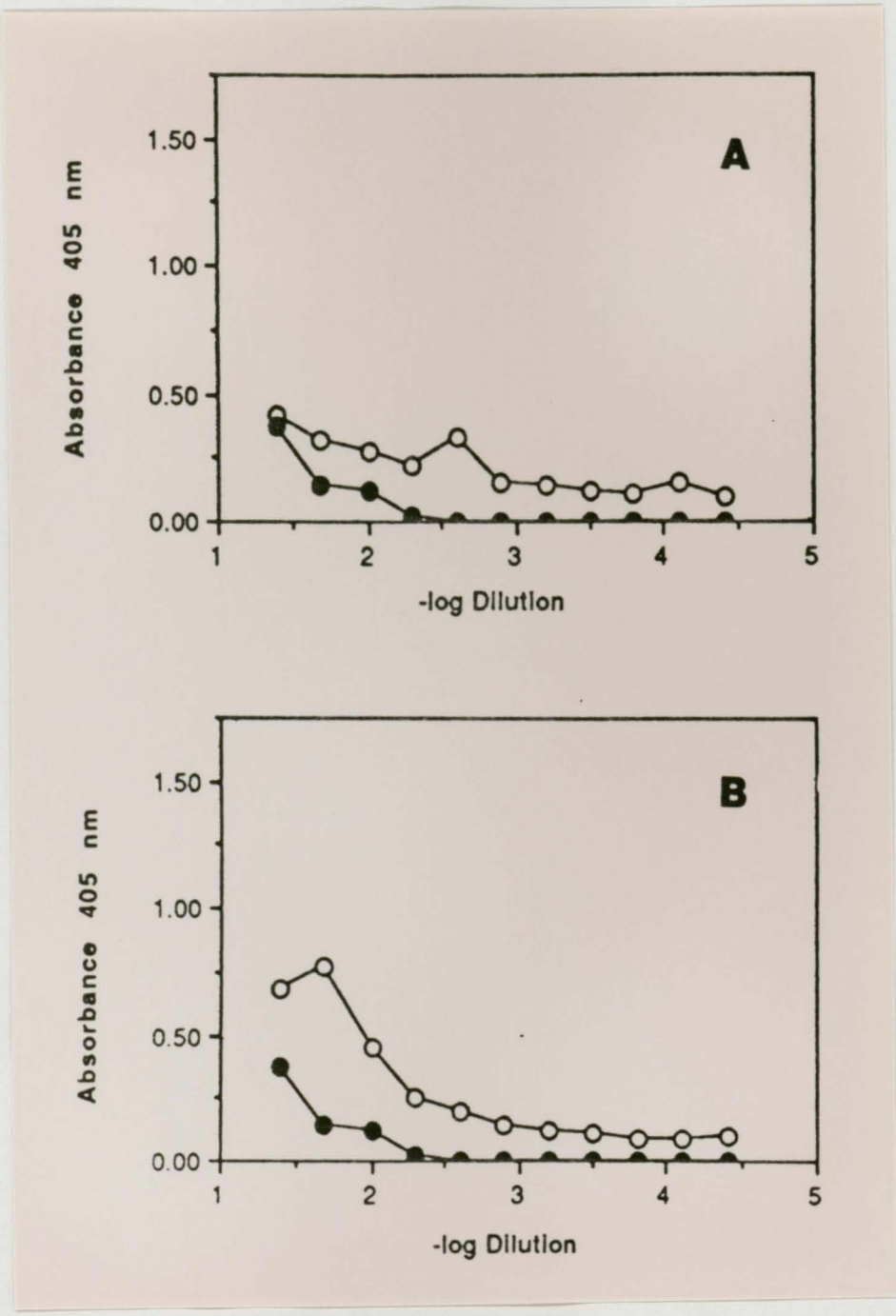


Figure 16. ELISA of polyclonal antiserum JB2 with whole cells of **A**. *S. typhimurium* (○) **B**. *S. enteriditis* (○). Normal Rabbit Serum (●).

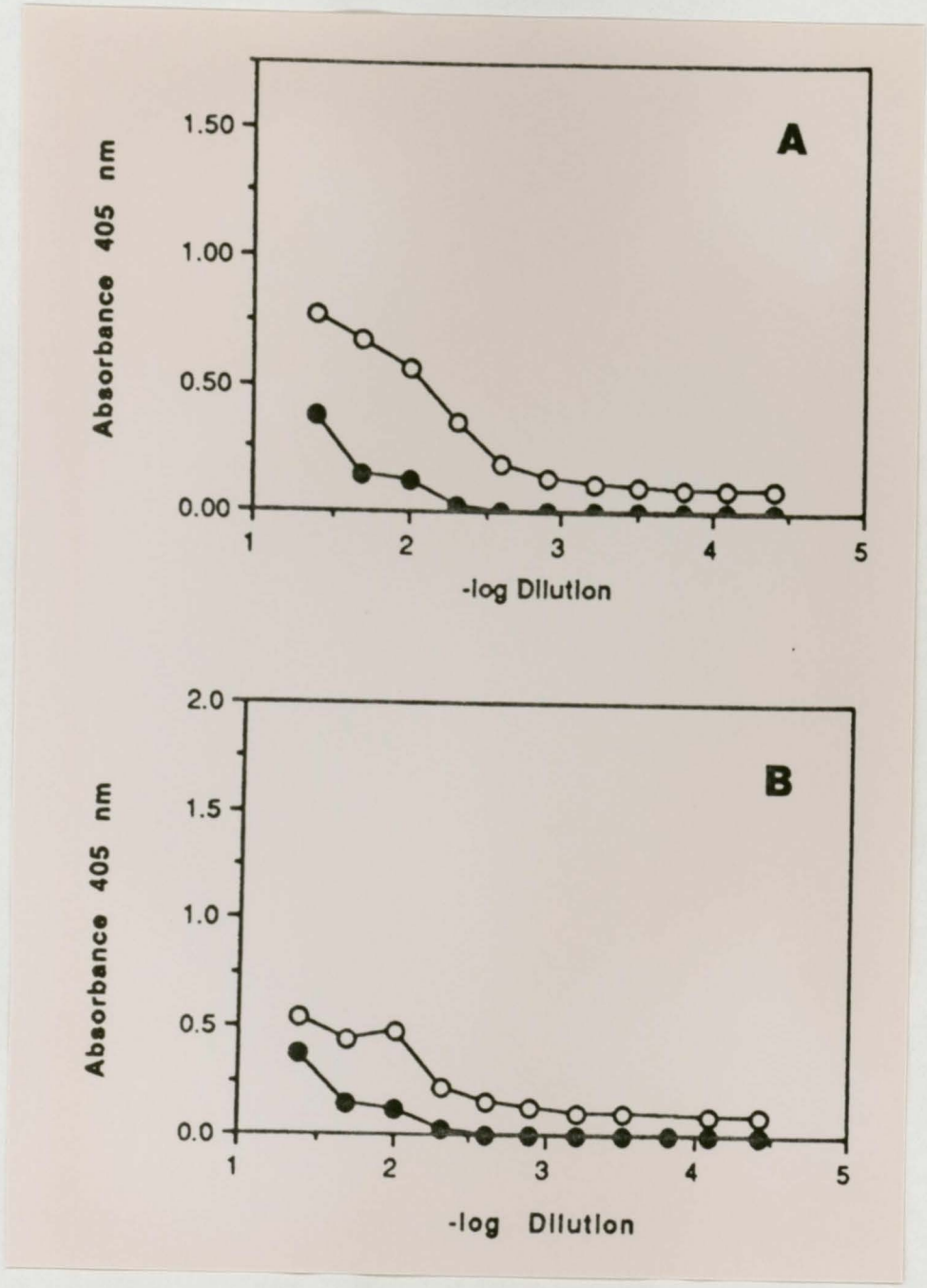


Figure 17. ELISA of polyclonal antiserum JB2 with whole cells of **A**. *E. coli* (○) **B**. *A. hydrophila* TF7 (○). Normal Rabbit Serum (●).

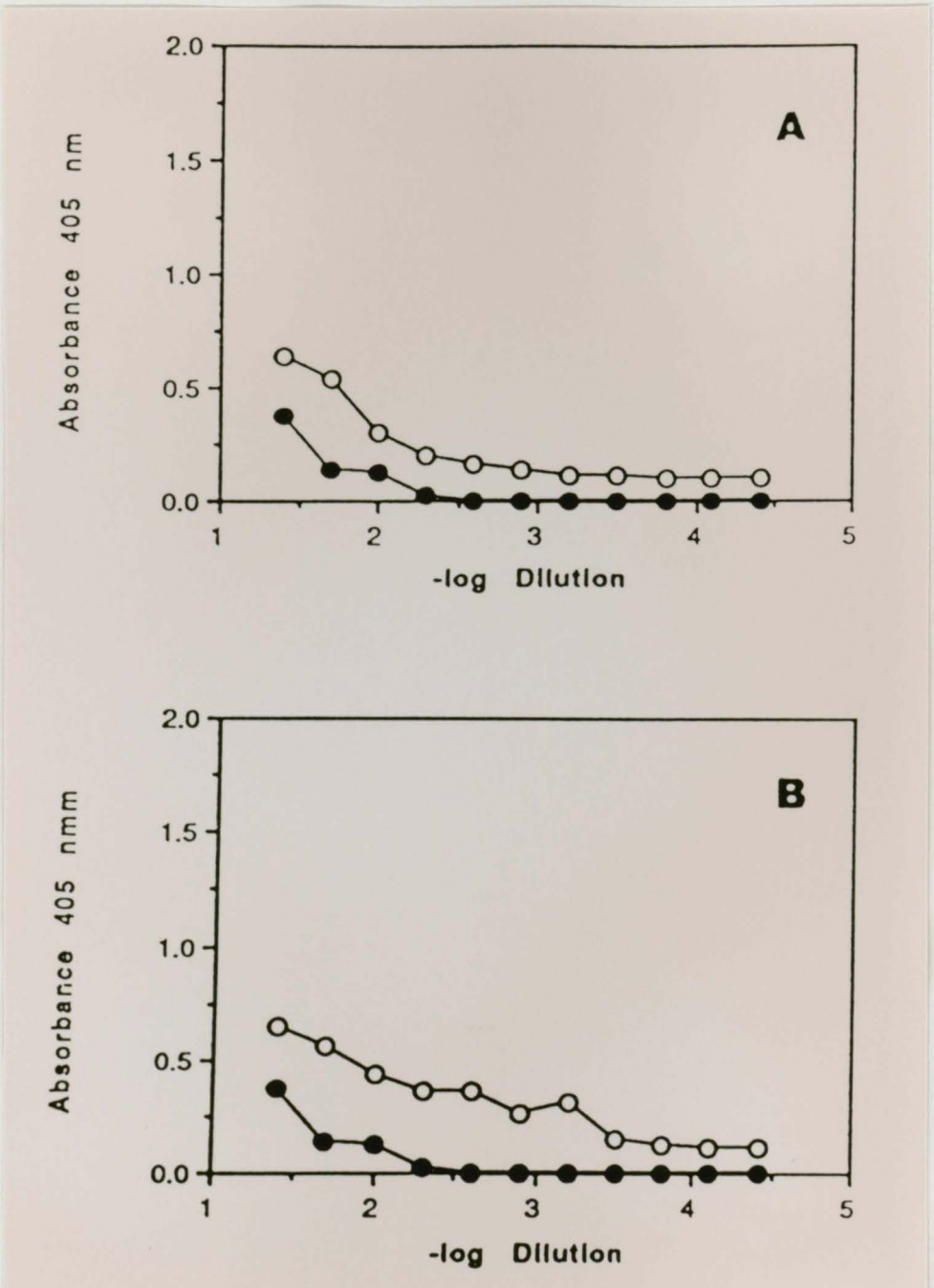


Figure 18. ELISA of polyclonal antiserum JB2 with whole cells of **A**. *C. jejuni* VC74 (○). **B**. *C. coli* VC167B (○). Normal Rabbit Serum (●).

flagellins of *C. pylori* strains 5442 and 5155 (Figure 13 lanes 4 and 5), and the flagellins of *C. jejuni* VC74 and *C. coli* VC167B (Figure 13 lanes 2 and 3). The antigenic cross-reactivity did not extend to the flagellin of *S. typhimurium* however (Figure 13, lane 1). While signal strength differences were not apparent in the immunoblot analysis, ELISA showed that purified *C. jejuni* VC74 flagellin provided a stronger signal (as measured by absorbance) than purified *C. coli* VC167B flagellin (Figure 14 A and B). ELISA with antiserum JB2 further showed that although flagellin could be detected on dried cells of heterologous strains of *C. pylori* 5155 and 5442 (Figures 15A and 15B), no reactivity was observed when whole flagellated cells of *S. typhimurium, S. enteritidis* (Figures 16A and B), *E. coli, A. hydrophila* (Figures 17A and B) were used as antigens. These results provided additional evidence for the absence of cross-reactive epitopes on the flagella of these other gram-negative bacteria. The reason the cross reactivities of *C. jejuni* and *C coli* (Figures 18A and 18B) with antiserum JB2 are low is because the cross reactive epitope on the filaments of *C. jejuni* and *C. coli* is internal and thus not exposed as a result of drying the cells at 37 C overnight onto an ELISA plate.

Indirect immunofluorescence. To evaluate the extent to which the inner flagellin filament was exposed in a population of *C. pylori* cells, indirect immunofluorescence assays were performed. Cells of a motile culture of *C. pylori* 5294 were gently fixed with methanol, incubated with polyclonal antiserum JB2 and then incubated with fluorescein labelled goat anti-rabbit antiserum. As can be seen in Figure 19A, very few flagellar filaments displayed immunofluorescence, even in a dense smear of *C. pylori* cells. Similar results were obtained with antiserum JB3 (data not shown).

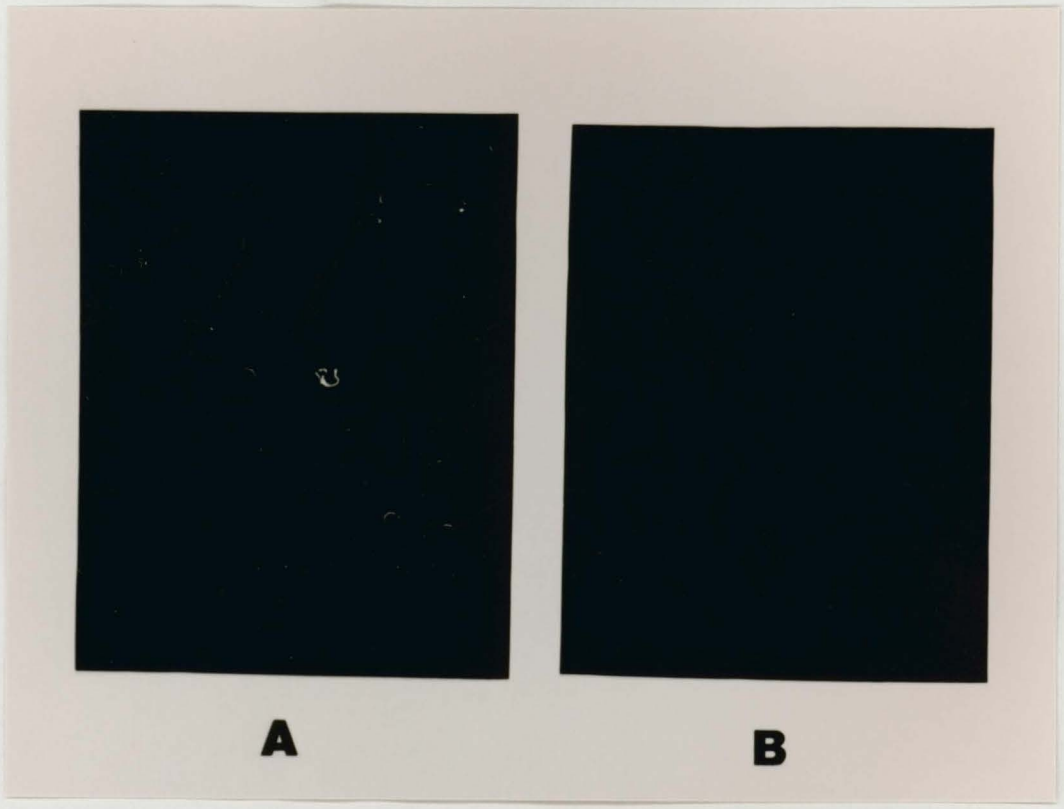
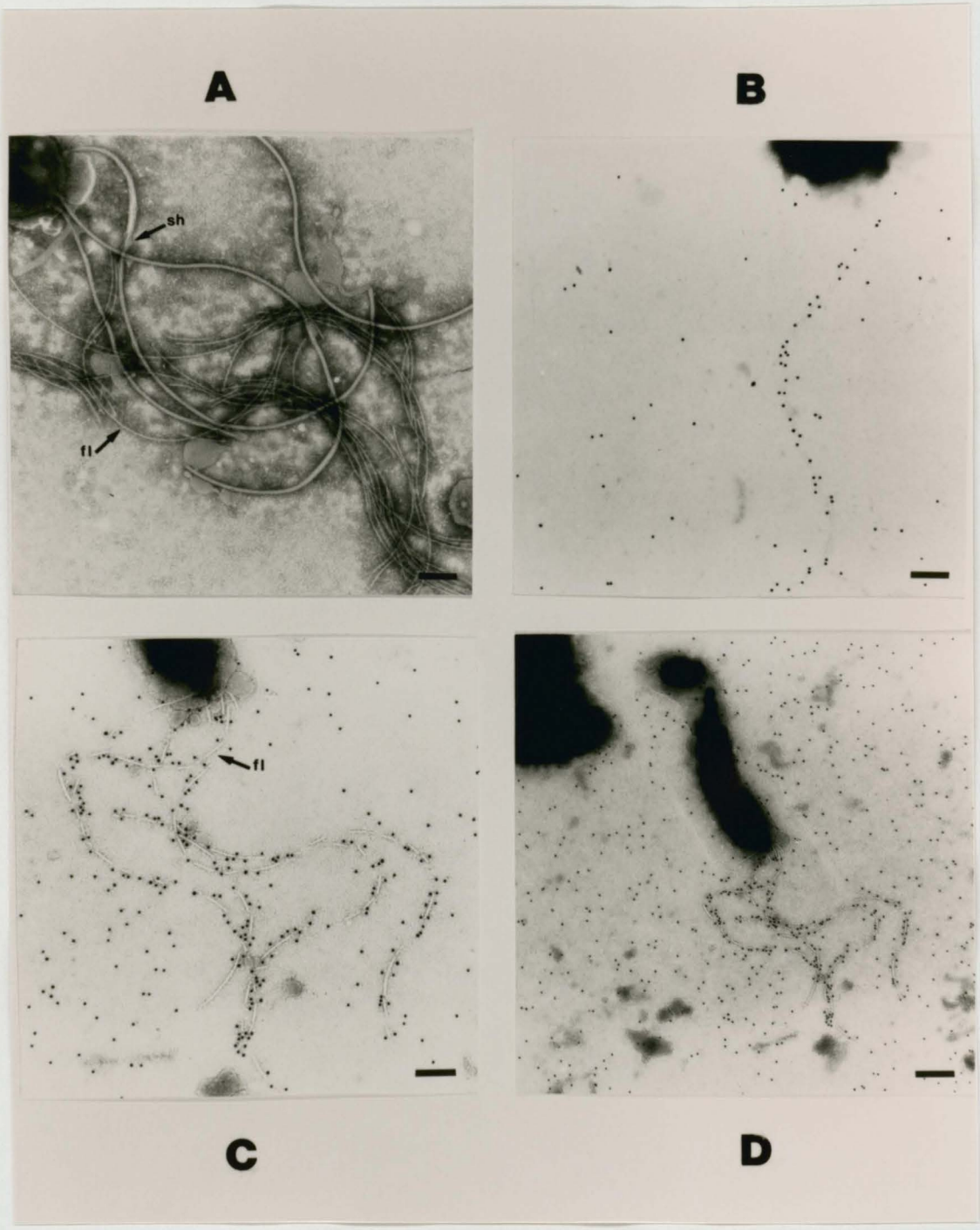


Figure 19. Indirect immunofluorescence assay of whole cells of *C. pylori* 5294 with polyclonal antiserum JB2. **A**. Immunofluorescence of unsheathed portions of flagella filaments of *C. pylori* 5294. **B**. Cells incubated in absence of antiserum.

Figure 20 Immunogold labelling of *C. pylori* 5294 with polyclonal antisera JB2 and JB3 diluted 1:10. **A**. Flagella showing sheathed flagellar filaments (sh) and unsheathed flagellar filaments (fl) negatively stained by 2% PTA, pH 7.2. Bar indicates 120 nm. **B**. Unsheathed flagellar filament labelled with antiserum JB3. Bar indicates 120 nm. **C**. Unsheathed flagellin filaments labelled with antiserum JB2. Bar indicates 180 nm. **D**. Unsheathed flagellin filaments labelled with antiserum JB2. Bar indicates 300 nm.

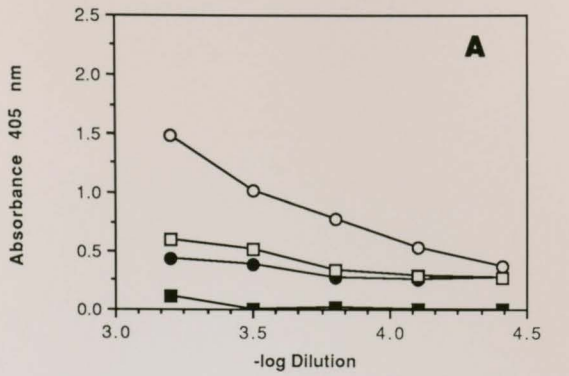

A
B
sh
fl
fl
C
D

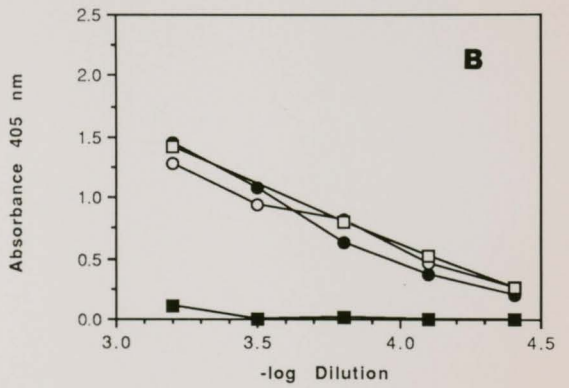
This immunofluorescence analysis provided convincing immunological and morphological evidence that the protein filament of *C. pylori* flagella was masked by the outer flagella sheath, and showed that only a small number of flagella have damaged sheaths and exposed inner flagellin filaments.

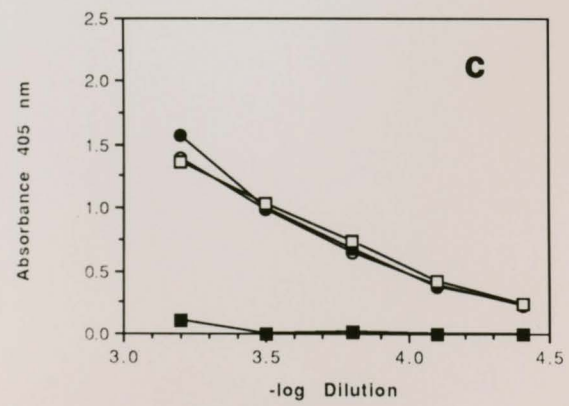
Immuno-electron microscopy. Immunogold labelling experiments were performed to confirm the immunofluorescence results. Electron microscopic examination of *C. pylori* 5294 cells reacted with either of the polyclonal anti-*C. pylori* flagellin antisera showed that antibodies bound to unsheathed flagellin filaments, but not to sheathed flagella. Typical results are shown in Figure 20. Figure 20A shows that the difference in thickness between a sheathed flagellar filament and an unsheathed flagellar filament is readily apparent in a negatively stained preparation. Figure 20B shows the immunogold labelling along an unsheathed flagellar filament of *C. pylori* 5294 by polyclonal antiserum JB3. Figure 20C similarly shows the immunogold labelling of unsheathed flagellar filaments of *C. pylori* 5294 by polyclonal antiserum JB2, and Figure 20D confirms the inability of this antiserum to label the surface of the *C. pylori* 5294 cell.

Absorption studies. To provide additional evidence that the flagella sheath did not contain the same 58 kDa protein that comprised the flagellar filament, cell absorption studies were performed. When antiserum is absorbed with boiled cells of the homologous strain, antibodies to heat stable surface-exposed antigens (LPS) should be removed. Similarly when antiserum is absorbed with live, motile cells of the homologous strain, antibodies to both heat stable and heat labile (protein) surface antigens should be removed. Antibodies to non-exposed antigens should not be

Figure 21. ELISA after absorption of antibodies in polyclonal antisera by boiled cells and live cells of *C. pylori* 5294. **A**. Antigen is glutaraldehyde fixed whole cells of *C. pylori* 5294 reacted with antiserum JB1. **B**. Antigen is purified 58 kDa flagellin from *C. pylori* 5294 reacted with antiserum JB1. **C**. Antigen is purified 58 kDa flagellin from *C. pylori* 5294 reacted with antiserum JB2. (○) Unabsorbed control antiserum; (□) Antiserum absorbed with boiled cells; (●) Antiserum absorbed with live cells; (■) Normal Rabbit Serum.


A
Absorbance 405 nm
2.5
2.0
1.5
1.0
0.5
0.0
3.0
3.5
4.0
4.5
-log Dilution


B
Absorbance 405 nm
2.5
2.0
1.5
1.0
0.5
0.0
3.0
3.5
4.0
4.5
-log Dilution


C
Absorbance 405 nm
2.5
2.0
1.5
1.0
0.5
0.0
3.0
3.5
4.0
4.5
-log Dilution

removed by either absorption. ELISA analysis of the ability of antiserum JB1 to react with glutaraldehyde fixed cells before and after absorption confirmed that when the absorption procedure was applied to cells of *C. pylori* 5294, antibodies to surface antigens were indeed removed (Figure 21A). In contrast, when the antigen in the ELISA was purified flagellin, no decrease in antibody titer to flagellin was obtained upon absorption (Figure 21B) indicating that epitopes of the 58 kDa protein were not surface exposed. Had the sheath contained the same protein as the filament, antibodies to the 58 kDa protein should have been removed. A similar result was obtained in absorption experiments with anti-*C. pylori* 5294 flagellin antiserum. The results in Figure 21C show that the ELISA reaction of antiserum JB2 to purified flagellin was the same before and after absorption. Taken together these results provided further evidence that the *C. pylori* flagella sheath is composed of materials other than the 58 kDa flagellin, and the epitopes of the 58 kDa flagellin are masked by the sheath.

Monoclonal Antibody Analysis.

Of a total of 480 hybridomas, 111 reacted in ELISA with the immunizing antigen, Mono Q purified flagellin Of these, 32 clones proved to be stable and were tested for the ability to react in ELISA with glutaraldehyde fixed whole cells of several *C. pylori* strains, as well as with other *Campylobacter* species and *E. coli*, purified flagellin from *C. coli* VC167B, flagella filaments from *C. pylori* 5294 and *C. coli* VC167B. Reaction with bovine serum albumin (BSA) and human transferrin were used as negative controls. After a period of several weeks, three of these clones were stable and thus used to further characterize the immunological structure of the 58 kDa flagellin of *C. pylori* 5294. These clones, all of isotype IgG1, were 72c,

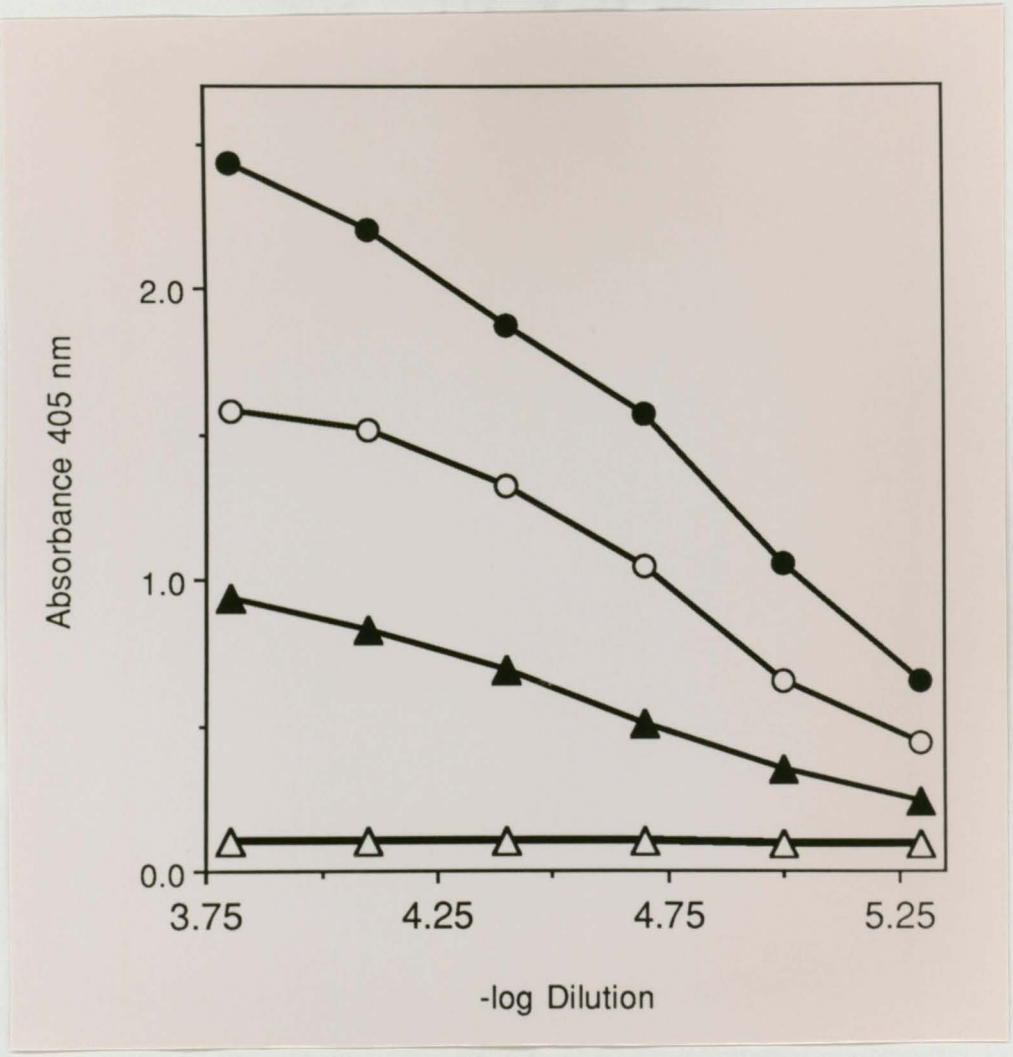
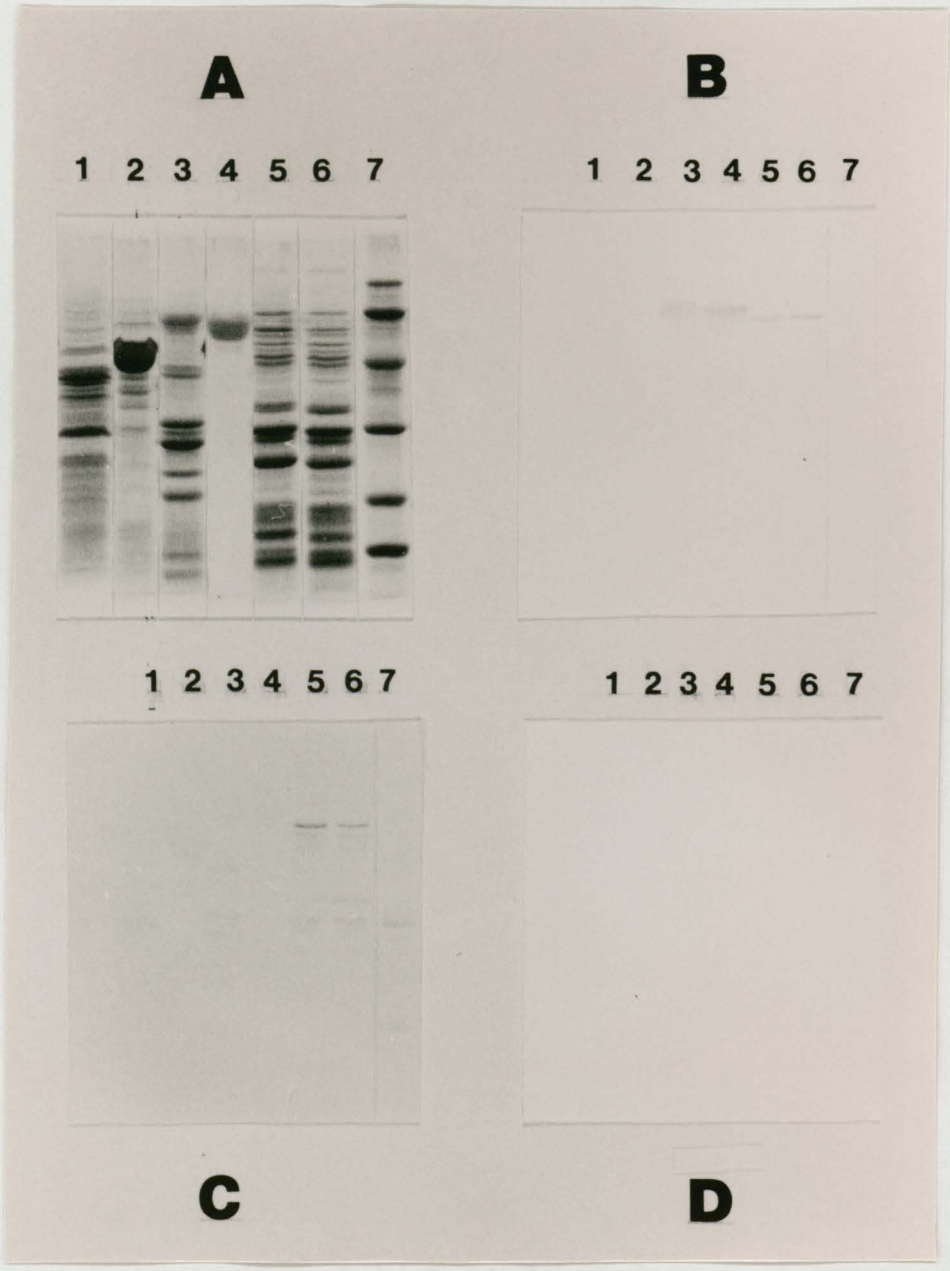


Figure 22. ELISA of hybridoma supernatants containing monoclonal antibodies 72c, 104a, and 220a reacted with purified *C. pylori* 5294 flagellin. (●) MAb 72c; (○) MAb 220a; (▲) MAb 104a; (△) Normal Mouse Serum.

Figure 23. Immunoblot analysis of antigenic cross reactivity of flagellins using monoclonal antibodies 72c, 104a, 220a diluted 1/10,000. **A**. SDS-PAGE stained by Coomassie blue. **B**. Immunoblot with MAb 72c. **C**. Immunoblot with MAb 220a. **D**. Immunoblot with MAb 104a. Lane 1, glycine extract of *S. typhimurium*; Lane 2, glycine extract of *A. hydrophila* TF7; Lane 3, glycine extract of *C. jejuni* VC74; Lane 4, purified flagellin from *C. coli* VC167B; Lane 5, glycine extract of *C. pylori* 5442; Lane 6, glycine extract of *C. pylori* 5155; Lane 7, low Mr standards, from top to bottom: 97.4, 66.2, 42.7, 31.0, 21.5, 14.4 kDa.


A
1 2 3 4 5 6 7
B
1 2 3 4 5 6 7
1 2 3 4 5 6 7
1 2 3 4 5 6 7
C
D

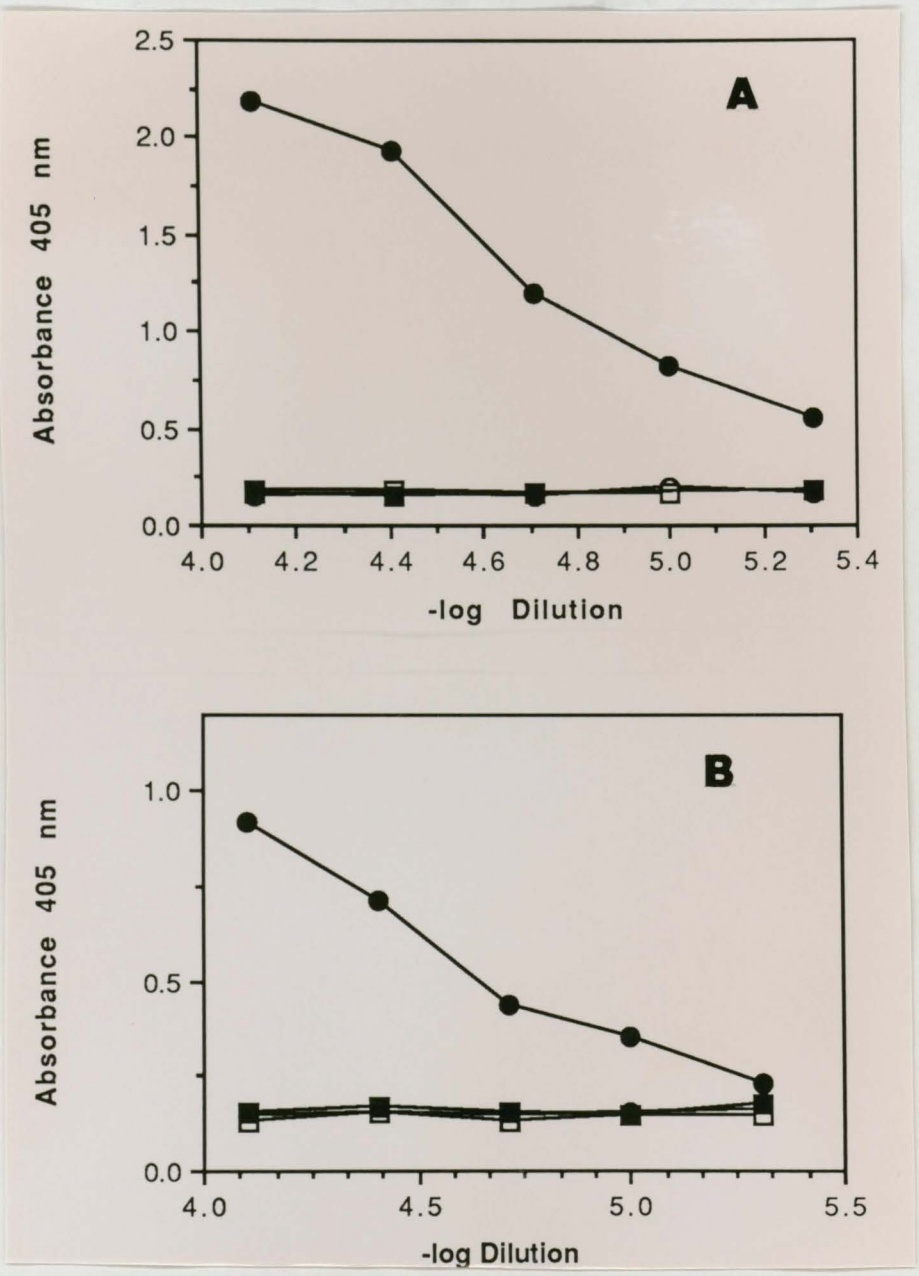


Figure 24. ELISA graphs quantitating the cross reactivity of monoclonal antibodies directed to the 58 kDa flagellin protein of *C. pylori* 5294. **A.** Antigen is purified flagellin from *C. jejuni* VC74. **B.** Antigen is purified flagellin from *C. coli* VC167B. MAb 72c (●); MAb 104a (■); MAb 220a (○); Normal Mouse Serum (□).

104 a and 220a. ELISA showed that all three clones provided high titer antibody when assayed against *C. pylori* 5294 flagellin (Figure 22). MAb 72c provided the strongest absorbance at all dilutions tested followed by MAb 220a, and MAb 104a.

The ability of the three MAbs to react in immunoblot assays with a variety of antigen preparations including whole cell lysates, glycine extracts and purified flagellin from several strains of *C. pylori*, and other *Campylobacter* species, and other flagellated gram-negative bacteria was tested. Figure 23B shows that MAb 72c reacted with the 58 kDa flagellin of both *C. pylori* strains tested, and with the flagellins of *C. coli* VC167B and *C. jejuni* VC74, but did not react with the flagellins of *A. hydrophila* or *S. typhimurium*. ELISA confirmed the cross reactivity of MAb 72c for *Campylobacter* flagellins (Figure 24). By comparison, MAb 220a appeared to be specific for *C. pylori* flagellin epitopes, reacting with both *C. pylori* flagellins tested but not with *C. coli* or *C. jejuni* flagellins (Figure 23C). In contrast, MAb 104a did not react in immunoblots with any of the samples tested (Figure 23D). However since ELISA analysis had shown that MAb 104a was capable of specific binding to *C. pylori* flagellin (Figure 22,), this result suggested that the epitope for this MAb was topographically assembled.

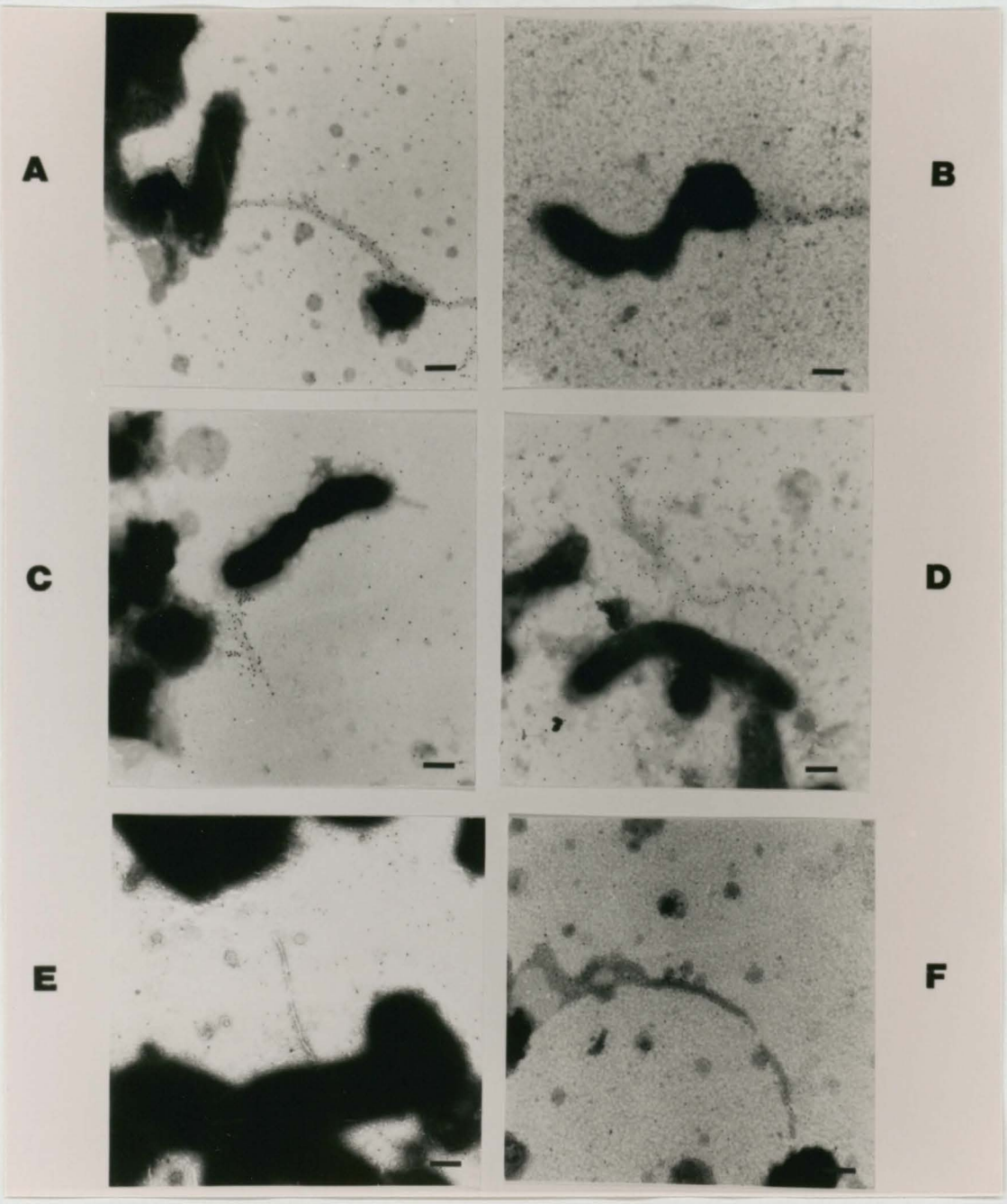
Surface immunolabelling techniques were then employed to provide further information on the epitopic structure of *C. pylori* flagella. Indirect immunofluorescence assay showed that each MAb bound to the surface of unsheathed *C. pylori* flagella filaments (Table 5). As had been the case when this technique was used with polyclonal anti-*C. pylori* flagellin antisera, only a small number of the filaments in a dense methanol-fixed smear of

Table 5. Monoclonal antibody reactions [a]

	ELISA	IMMUNO-BLOTTING	IMMUNOGOLD LABELLING	IFAT
MAb 72c *C. pylori* 5294	+	+	+	+
MAb 72c *C. coli* VC167B	+	+	-	ND
MAb 104a *C. pylori* 5294	+	-	+	+
MAb 220a *C. pylori* 5294	+	+	+	+

[a] + is a positive test reaction, - is a negative test reaction

Figure 25. Electron micrographs of immunogold labelling of unsheathed flagellin filaments of *C. pylori* with monoclonal antibodies diluted 1:50. **A**, *C. pylori* 5442 reacted with MAb 104a; **B**, *C. pylori* 5294 incubated with MAb 104a; **C**, *C. pylori* 5155 incubated with MAb 104a; **D**, *C. pylori* 5294 incubated with MAb 220a; **E**, *C. pylori* 5294 incubated with anti-mouse IgG1 antibody diluted 1/20 and with Protein-A gold conjugate diluted 1/20; **F**, *C. pylori* 5294 incubated with Protein-A gold conjugate diluted 1/20. Bar indicates 270 nm.


A
B
C
D
E
F

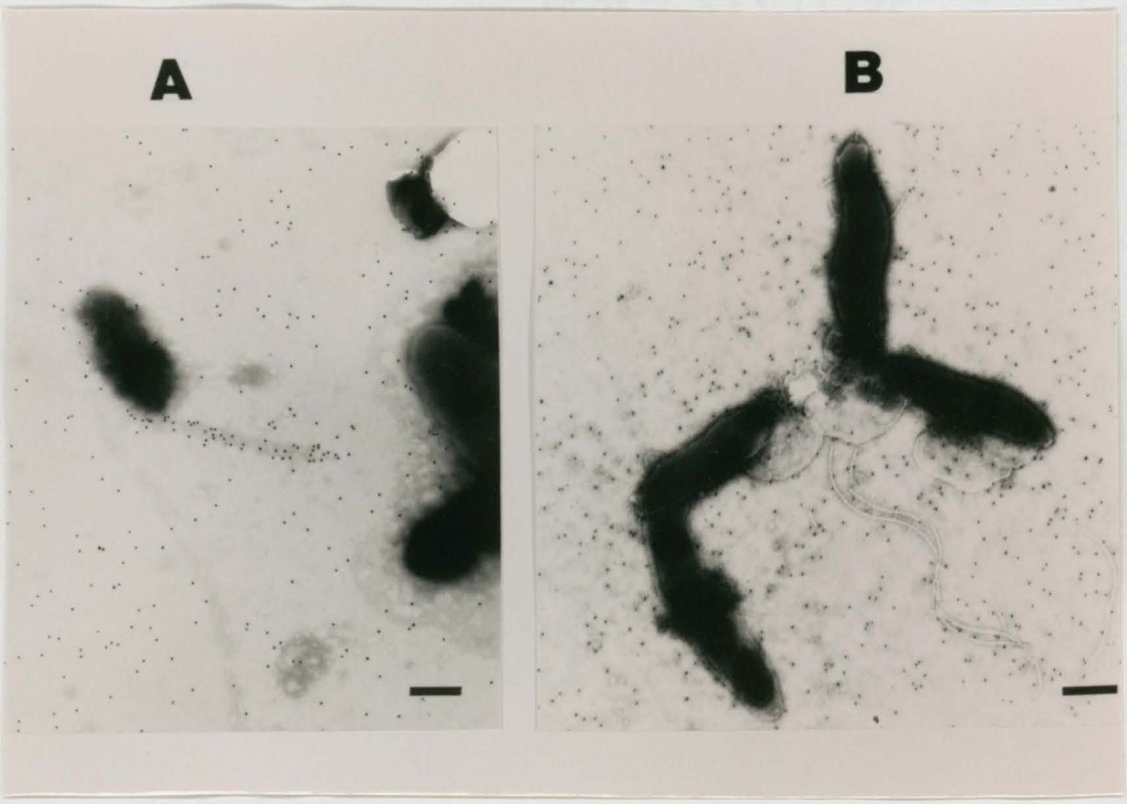


Figure 26 Electron micrographs of immunogold labelling with MAb 72c diluted 1:50. **A**, *C. pylori* 5294; **B**, *C. coli* VC167B. Bar indicates 233 nm.

Table 6. Summary of monoclonal antibody characteristics

MAb 72c	MAb 220a	MAb 104a
IgG1	IgG1	IgG1
Linear epitope on surface of flagellin filament	Linear epitope on surface of flagellin filament	Topographical epitope on surface of flagellin filament
Present on *Campylobacter* flagellins	Present on *C. pylori* flagellins	Present on *C. pylori* flagellins

C. pylori cells displayed unsheathed filaments. This result suggested that the epitopes for the three MAbs were located on the surface of the inner *C. pylori* flagellin filament. This was confirmed by immuno-electron microscopy. Figures 25A, 25B and 25C are electron micrographs showing immunogold labelling of the unsheathed flagellin filaments of *C. pylori* strains 5442, 5115, and 5294 with MAb 104a, while Figure 25D shows MAb 220a labelling the unsheathed flagellin of *C. pylori* 5294. Control experiments showing the absence of labelling when cells were incubated only with the intermediate anti-mouse IgG1 antibody plus the Protein-A gold conjugate, and when cells were incubated with only the Protein-A gold conjugate are shown in Figure 25E. In contrast to ELISA with *C. coli* VC167B flagellin, the antibody did not bind to *C. coli* VC167 flagella in immunogold assays (Figure 26B). This result indicated that the flagellin filaments of the sheathed *C. pylori* flagella and the unsheathed *C. coli* flagella were assembled in a different fashion, with the epitope for MAb 72c being masked in the assembled *C. coli* filament. This was consistent with previous findings with polyclonal antiserum produced against *C. jejuni* flagellin that the conserved linear epitopes are not surface exposed on the flagella filament but are internal (80).

A summary of the MAb results is presented in Table 5. It would appear that the 58 kDa flagellin of *C. pylori* has at least three different epitopes. One of these is the epitope recognized by MAb 72c, and is a linear epitope which is cross reactive among the flagellins of various species of *Campylobacter*. This epitope is located on the surface of the assembled inner flagellin filament of *C. pylori*, but is not located on the surface of native *C. coli* flagella filaments. The second is the epitope recognized by MAb 220a. This epitope is linear, *C. pylori* species-specific and located on the surface of the flagellin

filament. The third epitope is that recognized by MAb 104a. This *C. pylori* species-specific epitope is also located on the surface of the inner flagellin filament, and is topographically assembled.

DISCUSSION

The objective of this investigation was to identify, purify and characterize the flagella protein of *C. pylori*. Flagellar filaments were isolated from *C. pylori* using two different procedures, shearing followed by CsCl density gradient ultracentrifugation and shearing followed by ultracentrifugation, pH 2.0 acid dissociation and pH 7.0 reassociation. This technique had previously been employed to isolate the unsheathed flagella protein from *C. jejuni* (80) and *C. coli.* (79). Using both isolation procedures for *C. pylori*, a protein of subunit Mr 58 kDa was isolated, and in both cases, this protein was shown to react in immunoblotting and by ELISA with antiserum SML2 produced against the flagellin of *C. jejuni.*

Although the CsCl gradient centrifugation procedure provided material which allowed the first morphological confirmation that the subunit Mr 58 kDa of *C. pylori* was flagellin, the protein isolated from the procedure could not be freed from all traces of trypsin and ribonuclease added during the isolation procedure, so the pH 2.0 acid dissociation method was chosen for preparative isolations throughout the investigation. Using this procedure, the purification of *C. pylori* flagellin proved to be markedly more difficult than the purification of *C. coli* flagellin. Certainly the purification was complicated by the presence of the multimeric enzyme urease which was readily released from the *C. pylori* cell surface by simple shearing. The 63 kDa urease subunit was also one of a number of proteins of similar subunit Mr to the flagellin monomer which contaminated flagellin preparations in the case of *C. pylori*, but were absent in the case of *C. coli.*

However when the sample of flagellar filaments and flagellin monomers isolated by acid dissociation was equilibrated in 10 mM TRIS, pH 6.2, and subjected to anion exchange chromatography under the conditions described earlier, flagellin did elute as a single peak with 0.1 M NaCl in 10 mM TRIS, pH 6.2. This method was reproducible and efficient (69.1% yield), and provided a protein which was free of LPS contamination. Most importantly, SDS-PAGE and N-terminal sequence analysis showed that the protein was purified to homogeneity.

Significantly, Geis et. al. (38) also experienced difficulties in the only other reported purification of *C. pylori* flagellin. These workers used differential centrifugation, sucrose density centrifugation, followed by a sizing column to isolate their protein. The subunit Mr 51 kDa suggested by Geis et. al. (38) for the protein they isolated is lower than the 58 kDa obtained in this study for the flagellin of *C. pylori* 5294. The reasons for this apparent discrepancy are not clear at this time, however the difference in subunit Mr may simply reflect strain to strain differences, or technical differences in Mr estimation. Unfortunately, Geis et. al. (38) provided no biochemical data to confirm that their protein was purified to homogeneity, or to confirm its identity as flagellin, or to allow for more detailed comparison of the two proteins.

The Mono Q anion exchange procedure employed here was simpler than the procedure outlined by Geis et al. (38), and certainly provided sufficient quantities of high purity flagellin to satisfy the needs of the study. However workers who require larger quantities of highly purified *C. pylori* flagellin should consider using a combination of the three procedures evaluated during the course of this investigation. The first step in such a

preparative purification protocol might be CsCl density ultracentrifugation, without the addition of trypsin and ribonuclease. This would be followed by pH 2.0 disassociation-differential ultracentrifugation, gel filtration through a Superose 12 column, and finally Mono Q anion exchange chromatography.

The subunit Mr of 58 kDa places *C. pylori* flagellin among the larger flagellins reported to date. For example, this size is comparable to the 59-63 kDa flagellins of two other mucous colonizing spiral bacteria, *C. jejuni* and C. *coli* (79), the 51-59 kDa *E. coli* flagellin (38), and the 48-58 kDa flagellin for *Salmonella* species (38), but is greater than the 43-53 kDa flagellin of *Pseudomonas aeruginosa* (38), the 40 kDa flagellin of *A. hydrophila* TF7 (T. Trust, personal communication), and the three flagellin subunits of *Caulobacter crescentus* at 29 kDa, 27.5 kDa and 25 kDa (129). When considered on the basis of the various classes of amino acid , the amino acid composition of *C. pylori* 5294 flagellin is characteristic of a typical flagellin. For instance, the 39% hydrophobic amino acid content of the *C. pylori* protein is comparable to the 39% content reported for *B. subtilis* (20), the 33-37% content reported for *C. jejuni* and *C. coli* (79), and the 35% content reported for *S. typhimurium* (60). Also noteworthy among the compositions reported for various flagellins, including the *C. pylori* protein, is the minimal content of proline and histidine residues, and the absence of the cysteine and tryptophan residues. In only two cases did the *C. pylori* flagellin appear to be somewhat different than other flagellins. These apparent differences were with *B. subtilis* in glycine content where the C. *pylori* protein contained 11.3% glycine compared to 6.1% in the case of *B. subtilis*

flagellin , and in tyrosine content where the *C. pylori* protein contained 1.3% tyrosine compared to 0.3% tyrosine in *B. subtilis* flagellin.

This is the first report of an N-terminal sequence for *C. pylori* flagellin. Using the Dayhoff algorithm (19), the N-terminal 20 residues of the 58K *C. pylori* 5294 protein and the *C. coli* protein have a mean homology score of 101 (values of >100 are considered highly significant). Sequence alignment showed that of the first 24 amino acids identified in the N-terminal sequence of the flagellin of *C. pylori* 5294, 10 amino acids were identical with the N-terminal sequence of *C. coli* VC167B flagellin. Of the remaining nonidentical residues, three replacements are conservative. For instance, there is a conservative replacement of isoleucine for valine in the *C. pylori* sequence at residue 4, a replacement of valine for isoleucine at residue 8, and a replacement of valine for alanine at residue 15. Not surprisingly, the *C. pylori* sequence was more closely related to the *C. coli* flagellin in this region of the molecule than it was with either the *Salmonella* or *Bacillus* flagellins. However in the first 24 residues, *C. pylori* flagellin did share seven identical residues with *Salmonella* flagellin, and five identical residues with *Bacillus* flagellin. Importantly, subsequent studies with the flagellin of another strain of *C. pylori* isolated in Sweden (Kostrzynska and Trust, personal communication) has confirmed that the sequence reported here appears to be conserved among *C. pylori* flagellins.

This biochemical evidence of the identity of the *C. pylori* flagellin throws some doubt on the earlier finding of Perez-Perez and Blaser (112), and Dunn et. al. (23). In 1987, Perez-Perez and Blaser (112) reported (although the data was not shown) that a 62 kDa protein of *C. pylori* cross reacted with anti-flagellar antiserum of *C. jejuni*. However, the same

authors reported in 1989 that repeat immunoblot analysis of SDS-PAGE gels (the data was not shown) failed to demonstrate this cross reactivity. In the same report, the authors also failed to show this cross reactivity with repeat immunoblot analysis of two dimensional gels of flagellar preparations of *C. pylori* when reacted with antiserum directed against *C. jejuni* flagella. Dunn, et. al. (23) subsequently reported that two dimensional gel electrophoresis of isolated flagellar preparations resulted in four charge trains: 1. pI 5.6-5.8, 66 kDa Mr, urease subunit; 2. pI 5.2-5.5, 63 kDa Mr, urease subunit; 3. Mr below 29 kDa; 4. 59-62 kDa Mr, pI near 6.0.

Dunn et. al. interpreted this last charge train as an artifact of preparation as no such spot was observed in the whole cell preparation. Immunoblot analysis of the four charge trains with antiserum directed against *C. jejuni* flagellin failed to react with any of the spots. The authors however reported cross reactivity in a whole cell preparation of *C. pylori* with antiserum directed against *C. jejuni* flagellin at a charge train of 4.7 corresponding to a protein of 57 kDa. As a result of these findings, Dunn and coworkers concluded that the cross reactive epitopes carried by Mr 55-60 kDa proteins of *C. jejuni* and of Mr 57-59 kDa proteins of *C. pylori* were not associated with flagella, and that the Mr 57 kDa, pI 4.7 protein was flagellin. This conclusion was not however confirmed by immuno-electron microscopy, or by biochemical analysis. The reason why these findings of Perez-Perez and coworkers differ from the findings reported in this thesis remain unclear. But, based on biochemical, immunological, and morphological evidence, the flagellin of *C. pylori* 5294 has a subunit Mr of 58 kDa, and a pI of 6.0.

Immunochemical analysis of this *C. pylori* 5294 flagellin confirmed earlier reports of the presence of shared epitopes among the flagellins of spiral mucous colonizing bacteria. The purified 58 kDa *C. pylori* 5294 flagellin reacted by immunoblotting and ELISA with polyclonal antiserum produced against the purified *C. jejuni* flagellin. Similarly polyclonal antisera and monoclonal antibody 72c produced against the 58 kDa flagellin of *C. pylori* 5294 cross reacted with flagellin proteins of *C. jejuni* VC74 and *C. coli* VC167B in Immunoblots and ELISAs, but not with the flagellin protein from *E. coli, S. typhimurium, S. enteriditis,* or *A. hydrophila.* Indeed while the conserved N-terminal sequence among the various flagellins suggests a common ancestral gene (80), the presence of shared antigenicity among the *Campylobacter* flagellins suggests that this family of flagellins may have evolved differently from other flagellins.

In addition to epitopes conserved among the flagellins of spiral mucous colonizing bacteria, MAbs 104a and 220a provided evidence that *C. pylori* flagellin carries species specific epitopes. These two MAbs reacted with the three *C. pylori* flagellins tested, but not with *C. coli* or *C. jejuni* flagellins. Subsequent studies with an additional ten *C. pylori* strains, as well as with *C. laridis* and *C. fetus* have confirmed these findings (Kostrzynska and Trust, personal communication). As with MAb 72c, the epitopes recognized by MAbs 104a and 220a were shown to be located on the surface of the inner flagellin filament, and were masked by the outer flagella sheath. As was the case with MAb 72c, MAb 220a was reactive by immunoblotting suggesting that these two antibodies recognize linear epitopes. In contrast, MAb 104a was only reactive by ELISA suggesting that this MAb is specific for a topographically assembled epitope.

Cell absorption studies, and immunofluorescence and immuno-electron microscope studies also provided evidence that the 58 kDa flagellin is not part of the flagellar sheath, but comprises the actual inner flagellin filament. Polyclonal and monoclonal antibodies directed against the 58 kDa flagellin protein of *C. pylori* bound only to unsheathed portions of the flagellin filament and not to the cell surface. Interestingly, the immunogold labelling studies showed that while MAb 72c bound to unsheathed flagellar filaments of *C. pylori*, the antibody did not bind to flagellar filaments of *C. coli* VC167B, even though MAb 72c did react with the flagellin protein of *C. coli* VC167B by immunoblotting and by ELISA. This finding can be explained by different conformations for the flagellin molecules in the flagella filaments, so that the epitope for MAb 72c is surface-exposed on the flagellin filament of *C. pylori* but is hidden in the flagella filaments of *C coli* VC167B.

The MAbs raised in this study should provide valuable probes for more detailed studies on the structure of both the *C. pylori* flagellin, and the other *Campylobacter* flagellins. For example, MAbs 104a and 220a should allow for *C. pylori*-specific sequences to be identified, and for the surface-located domain of the *C. pylori* flagellin to be identified. In addition, MAb 72c should allow the conserved *Campylobacter* flagellin sequences to be identified at the nucleotide coding level, because the DNA sequence of the *C. coli* flagellin gene has recently been established (78). This MAb can also be used to probe for surface domains in the case of the *C. pylori* flagellin, and for non-surface located domains in the case of *C. coli* flagellin. This information will contribute to our understanding of how *Campylobacter* flagellin monomers are assembled into the mature flagellin filament.

MAb 104a and 220a might also allow the development of a dot-blot ELISA based immunoassay for *C. pylori*. Certainly in conventional ELISA used in this study, polyclonal antisera were able to specifically identify *C. pylori* cells, presumably as a result of damage to the outer flagellin sheath and exposure of the inner flagellin filament during this assay procedure.

The polyclonal antisera, and the monoclonal antibodies obtained in this study will also provide valuable probes to assist in the molecular cloning of the *C. pylori* flagellin gene. Similarly, the N-terminal amino acid sequence of the flagellin will allow the design and construction of a mixed oligonucleotide probe to assist in the cloning in the likely event that *C. pylori* DNA will not be well expressed in a foreign host such as *E. coli*. The availability of a cloned *C. pylori* gene would allow the nucleotide sequence of the gene to be determined, and the nucleotide sequence coding for species-specific epitopes to be identified. These sequences might provide the basis for development of species-specific DNA based diagnostic procedures. Certainly, the DNA sequence would provide valuable information for the phylogenetic analysis of the evolution of this important motility organelle.

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