ISOLATION AND CHARACTERIZATION OF A FAMILY OF PORIN PROTEINS FROM *Helicobacter pylori*

by

MAURICE MICHAEL EXNER

B.Sc. University of Regina

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Department of Microbiology)

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

April, 1997

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of MICROBIOLOGY AND IMMUNOLOGY

The University of British Columbia
Vancouver, Canada

Date April 21, 1997

ABSTRACT

Porins are pore forming proteins found in the outer membranes of gram negative bacteria. They are involved in the transport of small, hydrophilic molecules across the membrane, and are thus important in the uptake of a variety of substances from the external environment. An attempt was made to identify porin(s) in the outer membrane of the gastric pathogen *Helicobacter pylori* in order to gain insight into the physiology of this organism. Growth studies were done in order to optimize large scale growth conditions for *H. pylori*, and a novel growth media was developed such that large masses of membrane proteins could be obtained. Five porin proteins, (HopA, B, C, D and E) were identified and purified. N-terminal sequence analysis revealed that all five shared a strong degree of homology with each other which indicated that this was a group of related proteins. Model membrane analyses showed that the HopA, B, C, and D proteins functioned as channels. They all were functional as monomers, and they possessed similar channel forming properties in that they formed channels with conductances between 0.24 and 0.36 nS in 1.0 M KCl pH 7.0. HopE also formed channels, but it showed different pore forming characteristics, as even though it still appeared to function as a monomer, it formed large channels with conductances of 1.5 nS in 1.0 M KCl. Since most porins previously isolated function as trimers, a series of major outer

membrane proteins which were isolated as monomers from a variety of organisms were examined for pore forming ability in order to further show precedence for the existence of functional monomeric porins. Six of these proteins functioned as porins, indicating that porins which are functional as monomers may be more common than previously thought. Growth studies utilizing a defined medium demonstrated that the expression of HopA, B, and C was regulated by the amount of iron in the medium, and the different expression levels of the porins appeared to affect the uptake of a number of different antibiotics. Degenerate PCR primers created from N-terminal and internal protein sequences allowed amplification of DNA sequences encoding the N-terminal portions of two of the *H. pylori* porin proteins, and these DNA sequences were used to identify *hopA* and *hopB* clones from a *H. pylori* chromosomal DNA library that was created. Hybridization with *H. pylori* chromosomal DNA indicated that the *hop*A and *hop*B genes are located at different loci on the chromosome.

TABLE OF CONTENTS

List of Tables Page

List of Figures

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Bob Hancock, and my supervisory committee for their advice and suggestions regarding my project. I would also like to thank my co-workers and members of the Department of Microbiology and Immunology who have shared their knowledge and friendship with me over the years. In particular, I express my thanks to Susan Farmer for putting up with five years of incessant whining. I also wish to acknowledge the support of my family and friends; your help and presence is greatly appreciated.

The financial support of the Natural Sciences and Engineering Research Council and of the Canadian Bacterial Diseases Network is also gratefully acknowledged.

INTRODUCTION

A) *Helicobacter pylori*

Helicobacter pylori is a curved to spiral Gram-negative bacterium that is a gastric pathogen which colonizes the stomachs of humans. It has been known for decades that a variety of spiral organisms are present in the stomach (Freedberg and Barron, 1940), but *H. pylori* was only first isolated and cultured in 1983 from patients with gastritis and peptic ulcers. It was originally classified as a *Campylobacter* species (Marshall *et al.,* 1984), but upon further characterization, including ribosomal RNA sequencing, and examination of phenotypic properties, the new genus of *Helicobacter* was proposed (Goodwin *et al.,* 1989). Since the proposal of this genus, at least 13 new *Helicobacter* species have been identified. *H. pylori* is a slow growing, fastidious organism that normally requires a microaerobic atmosphere for growth, and it is these factors that have made it difficult to culture. *H. pylori* normally grows *in vitro* as a slightly spiral form, but, after approximately 4 to 5 days of growth, the spiral shapes begin to turn into coccoid forms which appear to be viable but non culturable. It is not known whether coccoid cells represent a degenerate or just a dormant form, but to date, all attempts to resuscitate coccoid cells have failed.

There does not seem to be a natural animal reservoir for *H. pylori*, and it appears to be spread through person to person contact by either a fecal-oral or oral-oral route. *H. pylori* has been identified in fecal samples (Kelly *et al.,* 1994), and it also appears to be

viable in water for up to 30 days in some cases (Shahamat *et al.,* 1993). In addition, a high prevalence of *H. pylori* infection has been found in cases in which vegetables washed with fecally contaminated water were ingested (Hopkins *et al.,* 1992). On the other hand, *H. pylori* has also been found in dental plaque (Desai *et al.,* 1991). Thus, there is evidence for both fecal-oral or oral-oral transmission, but neither route has been positively confirmed to date.

Studies have shown that over 60% of the entire population may be infected with *H. pylori* (Gasbarrini *et al.,* 1995, Tamassy *et al.,* 1995), and because of the enormous clinical implications which ensue from this, a tremendous amount of research has been initiated to study this organism. Despite difficulties in working with the organism, a number of different contributors to virulence have been identified for *H. pylori.* In many cases, the exact roles of these factors in the process of infection are not fully understood. Some *H. pylori* proteins or structures which appear to be involved in virulence include: urease (Smoot *et al.,* 1990, Eaton *et al.,* 1991, Mobley *et al.,* 1991, Ferrero *et al.,* 1991); cytotoxins (Leunk *et al.,* 1988, Cover *et al.,* 1992); catalase (Hazell *et al.,* 1991); proteases (Sarosiek *et al.,* 1988); hemagglutinins (Evans *et al.,* 1988, Lelwala-Guruge *et al.,* 1993); and flagella (Eaton *et al.,* 1989).

The most studied and possibly the most significant of the *H. pylori* virulence factors appear to be the urease enzyme and the vacuolating cytotoxin. *H. pylori* urease is a multimeric structure composed of a 29-31 kDa subunit and a 61-68 kDa subunit which

combine to form a complex with a molecular mass between 300 and 625 kDa. It has a reasonably high affinity for urea, having a K_M of 0.3 to 0.8 mM (Mobley *et al.,* 1988, Dunn *et al.,* 1990, Evans *et al.,* 1991). It has been proposed that this enzyme helps the bacterium to overcome the acidity of the stomach by creating a "cloud" of ammonia around the organism (Goodwin and Worsley, 1993), and *in vitro* studies have directly shown that urease activity is required for the survival of *H. pylori* in an acidic environment (Marshall *et al.*, 1990, Perez-Perez *et al.*, 1992). The hydrolysis of urea could also provide a nitrogen source for the organism, and this would also increase CO_2 levels which may be beneficial to the organism's metabolism. Urease activity also has direct effects on the pathology of infection. For example, ammonia which is generated by urea hydrolysis can be toxic to gastric cells (Megraud *et al.*, 1992). It has also been proposed that the ammonia production and the resulting alkaline pH causes a back diffusion of hydrogen ions into the gastric mucous, and subsequently degradation of the mucous as well as damage to the underlying gastric cells (Hazell and Lee, 1986, Miederer and Grubel, 1996).

The production, by *H. pylori*, of a vacuolating cytotoxin and a cytotoxin associated protein has been shown to be important in pathogenesis (Ghiara *et al.,* 1995), since it appears that significant pathological conditions arise predominantly in strains which express the cytotoxin and the cytotoxin associated protein. In fact, *H. pylori* strains have been divided into two types, type I which express VacA, the vacuolating cytotoxin, and CagA, the cytotoxin associated protein, and type II, which do not express these proteins (Xiang *et*

al., 1995). The vacuolating cytotoxin is a large aggregate composed of VacA subunits which have a molecular mass of approximately 87 kDa (Cover and Blaser, 1992). CagA is a highly immunogenic protein which has a predicted molecular mass of 131,517 (Tummuru *et al.*, 1993). VacA can directly damage gastric cells by causing vacuolization of these cells, whereas CagA does not appear to have a direct effect on gastric cells; however, its high degree of immunogenicity contributes to an inflammatory response which may lead to tissue damage in patients with chronic infections. There is some question as to whether or not both VacA and CagA are required to cause peptic ulcers. CagA is associated with 93.4% of patients with peptic ulcer disease, while only 56.6% of these patients possessed antibodies to VacA (Weel *et al.,* 1996), and this would suggest that CagA plays a more important role in ulceration. VacA still seems to play some role in ulceration, as it may be involved in inhibiting gastric mucosal repair (Ricci *et al.,* 1996). In addition, Ghiara *et al.* (1995) have shown that inflammatory responses which lead to ulceration may not be directed primarily to CagA, but rather to other components that are expressed in CagA-containing strains.

H. pylori's niche is in the gastric mucous and underlying epithelium, and it often adheres very tightly to gastric epithelial cells (Kobayashi *et al.*, 1993). In some cases, it may also be present between epithelial cell junctions. It is a combination of factors, including this location of the organism in the body, the toxicity involved with the activity of the urease enzyme and the vacuolating cytotoxin, and the production of enzymes such as proteases that degrade the gastric mucous, which initiate clinical manifestations of disease. This,

combined with the host's inflammatory response lead to host cell damage and to the onset of gastritis and other disease conditions.

B) Clinical significance of *H. pylori*

Helicobacter pylori has been shown to be involved in a variety of disease conditions. Epidemiological studies have consistently related *H. pylori* infection with chronic gastritis (Goodwin *et al.,* 1987, Morris and Nicholson, 1987), dyspepsia (Marshall *et al.,* 1985, Morris and Nicholson, 1987), peptic ulcers (Buck *et al.,* 1986, Graham, 1989, Sipponen *et al.,* 1990), and gastric carcinomas (Fox *et al.,* 1989, Parsonnet *et al.,* 1991, Sipponen *et al.,* 1992). In addition, infection has recently been correlated with the development of coronary heart disease (Martin-de Argila *et al.,* 1995, Patel *et al.,* 1995). Many of the disease states induced by *H. pylori* infection generally start with gastritis, and the severity of the disease symptoms then depend on the development from an acute infection to a more prolonged chronic infection.

Gastritis, or inflammation of the gastric mucosa, may begin as an acute inflammation which occurs after infection. In some cases, infections may be asymptomatic; however, if the infection is not cleared by the immune response or by antibiotic therapy, this acute condition may progress to chronic gastritis which results in tissue atrophy and a loss of normal glands in the stomach. In turn, this may lead to gastric metaplasia, which is the growth of new gastric epithelium and glands which are less differentiated than the normal

tissues of these types. As the length of infection progresses, tissue atrophy and metaplasia generally become increasingly severe (Siurala *et al.,* 1985, Sipponen *et al.,* 1993). In many cases, *H. pylori* infection may also spread to the duodenum, which results in the same types of atrophy and metaplasia found in the stomach.

Chronic gastritis, which is nearly always associated with *H. pylori* infection (Marshall, 1990), is an important factor in the development of peptic and duodenal ulcer disease. In fact, nearly 100% of patients with chronic duodenal ulcer disease, and 80% of patients with chronic gastric ulcer disease are infected with *H. pylori* (Ormand and Talley, 1990). Tissue atrophy and mucous degradation are likely contributors to ulceration, but there are also a number of environmental conditions, including acid production and the genetic dispositions of the host, which also contribute to the formation of ulcers.

Gastric cancer is another condition which may not be solely caused by *H. pylori* infection, but the risk of developing gastric cancer is greatly increased with the presence of chronic gastritis. *H. pylori*-induced gastritis can lead to damage to the DNA of gastric tissues by the production of free radicals which are generated in the oxidative bursts associated with immune and inflammatory responses. In addition, gastritis is associated with a constant regeneration of epithelial cells, and this may allow for the enhancement and selection of transformed malignant cells, which would outgrow other clones (Sipponen *et al.,* 1993). These are mechanisms by which gastritis may lead to gastric cancer, and

although gastritis may not be the single cause of gastric cancer, it definitely contributes to the possibility of developing gastric cancer.

Once *H. pylori* is acquired, it may persist within a host for years, and possibly for life. Chronic *H. pylori* infections exist even though there appears to be an immune response to the organism. Considering the significant health risks that can be involved with chronic *H. pylori* infection, it has been desirable to develop antibiotic therapies to eradicate the organism from infected individuals. Eradication of the bacterium has proven to be quite difficult, however, and in many cases, recurrence of infection occurs. Although *H. pylori* is quite susceptible to a number of antibiotics *in vitro* (McNulty *et al.,* 1985, Lambert *et al.,* 1986), this susceptibility has correlated poorly with *in vivo* efficacy. There are a number of problems with using antibiotic therapy to eradicate *H. pylori.* First of all, even though an aggressive triple therapy (e.g. two antibiotics, such as amoxicillin and metronidazole, along with a proton pump inhibitor such as omeprazole) is often prescribed, this treatment is not always successful (Deltenre *et al.*, 1996, Seppala *et al.* 1992, Marshall *et al*, 1988). In addition, the treatment is very expensive, and compliance with some therapy regimens has been problem due to various side effects that occur with such therapy (Glupczynski *et al*, 1989). Another important factor contributing to the relative inefficiency of conventional antibiotics is the site of infection, which is the gastric mucosa. Many antibiotics will not be found in sufficient concentrations in the gastric mucosa, or they may be inactivated by the low pH of the stomach (Sabath *et al*, 1968, Glupczynski and Burette, 1990, Veldhuyzen *et*

al, 1992). Pathogen-dependent factors which influence antibiotic susceptibility are also significant, and among the most important factors are the permeability of the bacterium to a particular antimicrobial agent, the production of enzymes, such as β-lactamases which degrade antibiotics, and the presence of efflux pumps which extrude the antibiotic from the cell. However, none of these mechanisms have been demonstrated in *H. pylori* to date.

Despite the ongoing research into *H. pylori* pathogenesis, along with studies relating to treatment of *H. pylori* infection, there is still no simple cure for infection or for the diseases which are caused after infection. Many pathogenic determinants are now better understood, but there are still many fundamental questions that remain unanswered. For example, the exact mechanism by which *H. pylori* is transmitted is still unknown, as is the fate of coccoid cells. The fact that basic questions such as these have not been answered stresses the continuing need for the study of this organism.

C) Bacterial outer membranes

1) General

Since prior to this study little was known about the outer membrane of *Helicobacter pylori*, the outer membrane of the *Enterobacteriaceae* will first be discussed. In Gram-negative bacteria, the outer membrane provides a selective barrier between the cell and the external environment. It acts as a molecular sieve that allows the passage of molecules into and out of the cell, and it helps to maintain the structure of the bacterial cell. The outer

membrane is an asymmetric lipid bilayer which contains lipopolysaccharide (LPS) in the outer leaflet and phospholipids, predominantly phosphatidyl ethanolamine (Cronan Jr., 1979), in the inner leaflet. This bilayer contains many proteins which are either anchored in or traverse the bilayer. Closely associated with the inner surface of the outer membrane and with various outer membrane proteins is a layer of peptidoglycan which provides structural integrity to the cell. A schematic representation of a bacterial outer membrane is seen in Figure 1.

2) Lipopolysaccharide

The LPS molecules in the outer leaflet of the outer membrane are amphipathic molecules consisting of an O-antigenic polysaccharide and a core oligosaccharide region, which are hydrophilic, and a Lipid A portion which provides the hydrophobic component. The O-antigenic polysaccharide is a repeating unit of tri to pentasaccharide units that can vary in composition and in the number of repeating units. This polysaccharide, which is the immunodominant portion of the LPS, is only present on approximately 10% of all LPS molecules in a given bacterium (Hancock *et al.,* 1994). The core oligosaccharide, which usually consists of phosphates, heptoses, hexoses, and a unique sugar, 2-keto-3-deoxyoctonoate (KDO), is relatively conserved in a given bacterial species. The core region is linked to the Lipid A by the KDO, and the Lipid A is in turn inserted in the outer membrane by its fatty acid chains. Lipid A consists of 5 to 7 fatty acids which are O- and N-linked to a diglucosamine disaccharide which normally has phosphates at the 1 and 4

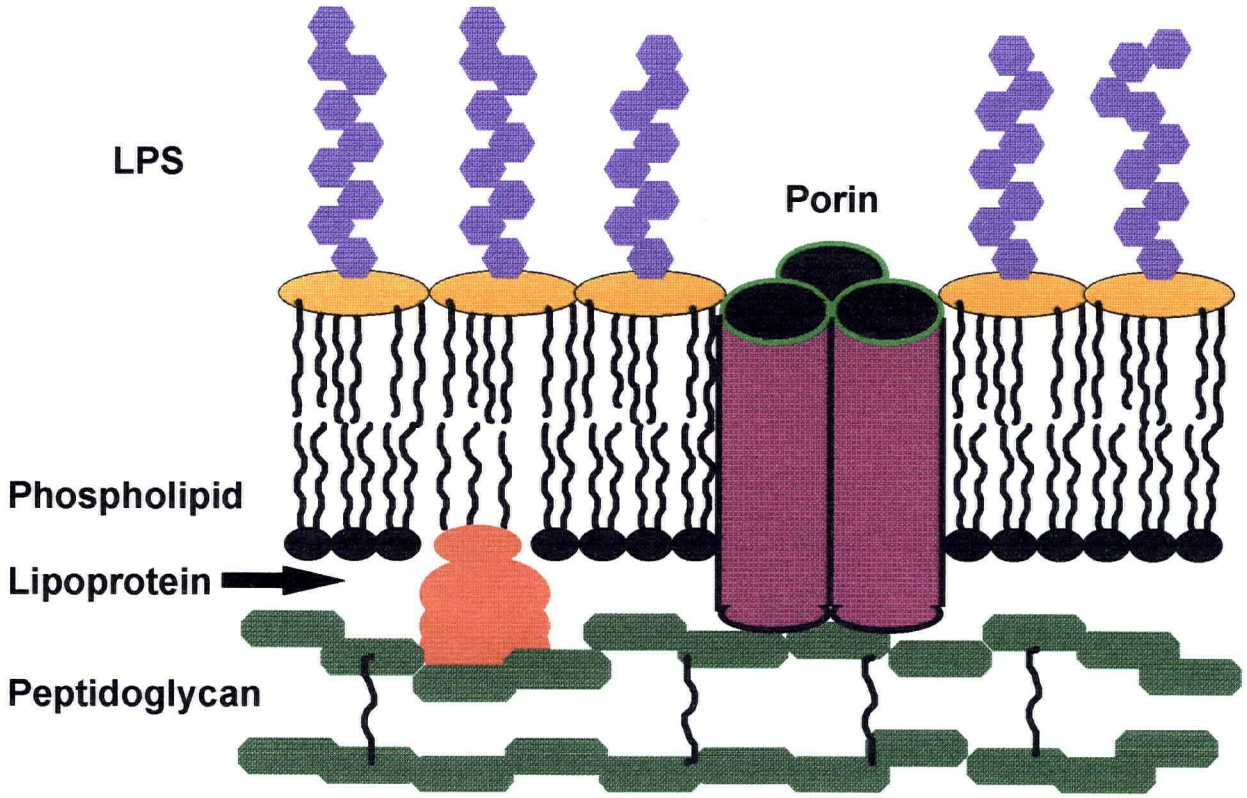

Figure 1. Schematic representation of an enterobacterial Gram-negative bacterial outer membrane and the underlying peptidoglycan layer.

positions. The Lipid A moiety is highly conserved in most bacterial genera, and it is responsible for endotoxicity.

LPS is often tightly associated with proteins in the membrane via non-covalent associations, and this may be important, in part, in maintaining outer membrane stability. LPS molecules also interact with each other, and divalent cations stabilize the LPS-LPS interactions by neutralizing the negative charges of attached sugar and phosphate groups. The crossbridging of LPS molecules with divalent cations is important in maintaining membrane stability. In addition to the structural role of LPS, its high surface negative charge provides a barrier function, as it results in a layer of tightly bound water and thus restricts the free passage of hydrophobic molecules through the lipid bilayer.

3) Proteins and lipoproteins

Proteins are also important constituents of the outer membrane, and they can be found associated with both the inner and outer leaflets of the outer membrane. One of the most abundant proteins associated with the outer membrane of *Escherichia coli* is the 7.2 kDa Braun's lipoprotein which is found both covalently (33%) or non-covalently (67%) associated with peptidoglycan (Braun, 1975). The protein portion of the Braun lipoprotein interacts with the peptidoglycan, while the lipid part is associated with the inner monolayer of the outer membrane, thus providing a part of the outer membrane structure. A number of other lipoproteins also exist in *Enterobacteriaceae*, including PAL (peptidoglycan associated lipoprotein) (Mizuno, 1979) which is non-covalently associated with the

peptidoglycan, and again, there is some evidence that PAL contributes to the structural integrity and barrier function of the outer membrane (Lazzaroni and Portalier, 1992).

Other types of outer membrane proteins contain no lipid moiety, such as OmpA from *Escherichia coli* and OprF from *Pseudomonas aeruginosa*. These proteins are completely embedded in the membrane, and they traverse the membrane in anti-parallel β-strands (Vogel and Jahnig, 1986, Rawling *et al.*, 1995). Studies using OmpA-deficient / Lpp (Braun's lipoprotein)-deficient and OprF-deficient mutants show that they have reduced membrane and cell stability, thus suggesting that these proteins play a structural role (Woodruff and Hancock, 1989). OmpA and OprF also appear to function as channels (Benz and Hancock, 1981, Sugawara and Nikaido, 1992), and OmpA has other functions; it can act as a phage and colicin receptor, and it can stabilize mating aggregates between recipients and F^+ donor cells (Skurray *et al.*, 1974, Morona *et al.*, 1984).

Although proteins such as OmpA and OprF function as channels, the non-specific trimeric porins of the porin superfamily are often the major proteins involved in transport of small substrates across the membrane of Gram-negative bacteria [i.e. substrates the size of β-lactam antibiotics or disaccharides]. As with the other membrane-associated proteins, porins also interact with membrane components. In particular, they have close associations with LPS molecules and with the peptidoglycan beneath the outer membrane. The structure and function of porin proteins will be discussed in sections D and E.

4) *Helicobacter pylori* outer membrane

H. pylori is a Gram-negative bacterium, so it possesses an outer membrane. However, its outer membrane has some distinguishing characteristics. Given the organism's unique gastric niche, it is not surprising to see what appear to be specialized features in its outer membrane. *H. pylori* LPS is unusual for a number of reasons. The LPS contains β-hydroxy stearic acid and stearic acid as major components (Geis *et al.*, 1990), and these fatty acids are not normally found in the LPS of other organisms. In addition, the Lipid A contains unusually long 3-hydroxy fatty acids (i.e., 3-hydroxyoctadocanoic and 3-hydroxyhexadecanoic) and the D-glucosamine backbone is phosphorylated at only the 1 position and not at the 4' position (Mattsby-Baltzer *et al.,* 1992, Muotiala *et al.,* 1992). The O-antigenic region of *H. pylori* LPS is unique in that its structure mimics Lewis blood group antigens (Aspinall *et al.*, 1996, Moran, 1996, Appelmelk *et al.*, 1996). This may act to hide the organism from the immune system, but on the other hand, it could also lead to the development of autoimmune diseases in the host. *H. pylori's* unusual LPS structure and composition may be why its LPS is up to 1000 times less toxic as an endotoxin than the LPS of typical enteric bacteria (Geis *et al.*, 1990, Mattsby-Baltzer *et al.,* 1992). In addition to the unusual fatty acids associated with LPS, *H. pylori* lipids include cholesteryl glucosides (Hirai *et al.,* 1995), which are not normally associated with either animal or bacterial cells. The exact function of these molecules is unknown, but they must have some effect on the physicochemical properties of the membrane (Haque *et al.,* 1995).

Another distinctive characteristic of the *H. pylori* outer membrane is that it appears to be either very closely associated with the inner membrane, or it has a density which is very similar to the inner membrane, because it is very difficult to separate the two membranes from each other. Sucrose gradient centrifugation generally separates membranes on the basis of density, and outer membranes are generally more dense than inner membranes because of their LPS and protein content. Attempts to separate inner and outer membranes of *Campylobacter* species (which are taxonomically related to *H. pylori*) by sucrose gradient centrifugation have not been successful (Page and Taylor, 1988), and this appears to true for *H. pylori* (Goodwin and Worsley, 1993). Regarding its protein content, *H. pylori* does not possess any highly expressed major outer membrane protein species, such as the porins that are present in most other Gram-negative bacteria, (Megraud *et al.*, 1985, Newell, 1987, Perez-Perez and Blaser, 1987, Dunn *et al*, 1989) and this may result in a less dense outer membrane.

D) Porin proteins

Porins are pore forming proteins which are found in the outer membranes of Gram-negative bacteria. These proteins serve as diffusion channels which facilitate the passage of hydrophilic molecules, below a given exclusion limit, across the bacterial outer membrane. Studies have shown that the size of porin channels generally ranges from 1-2 nm in diameter (Benz, 1985, Hancock, 1986, Nikaido, 1992), and this would allow the diffusion of most

growth requirements, including sugars, amino acids, peptides, and ions. Porins have an important role in infections, because, in addition to transporting nutrients, they provide the major route for the passage of certain classes of antibiotics across the outer membrane. It is known that most β-lactam antibiotics use primarily the general diffusion channels of porins to cross this membrane (Nikaido, 1988, Livermore, 1988, Yoshimura and Nikaido, 1985). In contrast, OprD from *Pseudomonas aeruginosa* actually contains a specific binding site for imipenem, thus allowing facilitated diffusion of this β-lactam antibiotic (Trias and Nikaido, 1990). Bacterial mutants which are resistant to β-lactam antibiotics can arise in the clinic, and can be the result of a mutation which decreases or eliminates the expression of a porin protein that was involved in the uptake of the antibiotic (Hashizume *et al.*, 1993, Hancock and Bell, 1988, Aronoff, 1988).

Porin proteins are typically functional as homo-trimers. Individual subunits normally have molecular masses ranging from 26,000 to 48,000 Daltons. They usually have an acidic pI, and they possess a high proportion of β-sheet structure (Lugtenberg *et al.,* 1983, Benz, 1985, Hancock, 1986). Many porins are found in very high copy number in a cell, with up to 10^5 per cell. The expression of porins is often regulated by environmental conditions, and a number of such regulated porins are listed in Table I. Many porins may also act as phage and colicin receptors (Table I), and this again is unsurprising because of their high levels of expression and their cell-surface exposure.

There are two basic classes of porins, the general-diffusion porins, and the substrate specific porins. General diffusion porins have no specific substrate or substrate binding site, and as the name implies, they allow for diffusion of hydrophilic molecules that fit through the most constricted portion of the channel. They may, however, be somewhat ion-selective due to the electrostatic properties of the amino acids lining the channel. Specific porins are those with a saturable, substrate-specific binding site within the channel, and they are often components of uptake systems. The crystal structure of the maltodextrin specific porin, LamB, from *E. coli* has shown that a substrate binding site is formed by amino acid residues in the β-barrel wall along with a protein loop which is folded into the channel to form a constriction site (Schirmer *et al.,* 1995), and a similar structure may exist for other specific porins.

E) Structure of porin proteins

Early studies on porin structure had suggested the presence of a stable β-barrel conformation, but a complete elucidation of porin structure and a confirmation of previous theoretical structures has only recently become available through the use of X-ray crystallography, which has provided structures for five porins (Weiss *et al.,* 1991, Cowan *et al.,* 1992, Kreusch and Schulz, 1994, Schirmer *et al.*, 1995). Crystal structures of *Rhodobacter capsulatus* porin (Weiss *et al.,* 1991), *Rhodobacter blastica* porin (Kreusch and Schulz, 1994) and of OmpF and PhoE from *E. coli* (Cowan *et al.,* 1992) show that all

Table I. Examples of porin proteins regulated by environmental conditions.

Bacterium	Protein	Growth Conditions Favoring expression	Involved as Receptor for Colicin/Phage	Reference
Escherichia coli	OmpC	High osmolarity	TuIb,Mel,T4,P A-2,434	Brass, 1986
Escherichia coli	OmpF	Low osmolarity	TuIa,T2,TP1	Brass, 1986
Escherichia coli	PhoE	Phosphate limitation	TC23,TC45	Brass, 1986
Escherichia coli	LamB	Presence of maltose	λ,K10,TP1	Brass, 1986
Pseudomonas aeruginosa	OprB	Glucose		Hancock, 1986
Pseudomonas aeruginosa	OprP	Phosphate limitation		Hancock, 1986
Xenorhabdus nematophilus	OpnP	Stationary growth phase		Forst *et al.*, 1995

of these porins have similar structures. Even though porins are present in the hydrophobic environment of the bacterial outer membrane, they have no long stretches of hydrophobic amino acids which could span the membrane. Rather, they possess amphipathic sequences and contain many charged residues. In addition, they do not contain regions of α-helices which may interact with the membrane, but instead, these porins form consecutively-folded, antiparallel β-sheets which create a very stable β-barrel structure. These four structurally characterized porins and many of the known functional porins are composed of 3 monomeric units which bind together non-covalently to create a stable trimeric structure. Specifically looking at the structure of the *E. coli* porins, the β-barrels of OmpF and PhoE are formed from 16 antiparallel β strands which are at a 35° to 50° tilt relative to the barrel axis (Cowan *et* al., 1992). The pore itself is also off-center, and it is at an angle of 16° relative to the barrel axis. There are long external loops between β-strands, and relatively short hairpin loops which face the periplasm. The amino and carboxy termini of the porins are linked by a salt bridge, and this helps to maintain the cylindrical barrel structure. A surface loop (loop 3, which is formed between β strands 5 and 6) is folded into the pore, and it contains 1.5 turns of an α-helix which constricts the pore. Loop 2 interacts with the loops of adjacent monomers, and, together with approximately one third of the barrel wall, forms salt bridges and hydrogen bonds which enable the trimeric porin structure to be held tightly together. There is also tight packing of large hydrophobic amino acids along the symmetry axis of the trimer, along with various hydrophobic interaction between strands of

different monomers, and this also contributes to trimer stability. In fact, 35% of the surface of a monomeric porin unit is involved in binding with adjacent monomers, so it is, perhaps, not surprising that the trimeric structure is stable to heating and to strong detergents such as sodium dodecyl sulphate.

The strands of the β-barrel that interact with the membrane are amphipathic (approximately), but nonetheless these strands still interact with the lipid bilayer membrane. Generally speaking, alternating hydrophobic and hydrophilic residues are oriented such that the hydrophobic amino acid side chains face outwards towards the fatty acids of the membrane. They also interact with hydrophobic residues on adjacent transmembrane protein strands, or they may fold over the mouth of the channel to constrict the entrance of the channel. The exterior walls of the β-barrel contain a 25 Å band of protruding hydrophobic side chains, mostly from aromatic residues, that face the membrane, and this band spans the approximate distance required to cross the hydrophobic core of the lipid bilayer. Meanwhile the side chains from hydrophilic residues face into the lumen of the channel, which is hydrophilic, and some are involved in interactions with loop 3 residues within the channel.

For a molecule to pass through the *E. coli* OmpF porin channel, it would have to fit through a constriction of approximately 7 Å x 11 Å. The pore diameter created by the β-barrel has been proposed to be approximately 15 Å x 22 Å (Cowan *et al.*, 1992), but the entrance to the channel is partially constricted to 11 Å x 19 Å by surface loops, and the inner channel is constricted to 7 Å x 11 Å by the insertion of loop 3 into the pore.

Hydrophobic molecules may pass through the channel, but because they would be surrounded by a structured "cage" of water (Nikaido, 1992), they would not pass as easily as hydrophilic molecules.

The structure of a substrate specific porin, LamB from *E. coli* is quite similar to that of the *Rhodobacter* porins and of OmpF and PhoE, except that it forms a β-barrel that is composed of 18 β-strands (Schirmer *et al.*, 1995). It too has a constriction formed by loop 3. This porin, however, has additional features which could contribute to the substrate specificity. For instance, the channel contains what has been termed a "greasy slide" (Schirmer *et al.*, 1995) which is composed of a series of aromatic residues within the channel that form a left-hand helical pathway from the entrance to the end of the channel. It is believed that the stacking of sugars between the aromatic residues could guide sugar molecules into the channel, and various charged amino acid residues within the channel could react with hydroxyl groups on the sugars, and this may be the cause of the stereospecificity of molecule selection, as well as the preference for tetrasaccharide over disaccharide binding.

Although the structures of different porin proteins are not identical, the basic β-barrel structure is conserved amongst monomeric subunits, and each subunit forms a separate pore that spans the membrane. Despite the basic conservation in structure, there is still not a substantial amount of similarity between most porin protein sequences. However, there appears to be a "superfamily" of related non-specific porins (Jeanteur *et al.,* 1991),

and Gibbs motif sampling also shows that sequences of some of the transmembrane β-strands of different porin species appear to be conceptually related (Neuwald *et al.,* 1995). In addition to sequence identity, many porins also show antigenic cross reactivity (Hofstra and Dankert, 1979, Singh *et al.,* 1992).

The strong interactions between monomeric porin subunits along with the β-sheet structure lends to the extreme stability of porins. Some porins require heating to 70°C in sodium dodecyl sulfate (SDS) before their stable structures can be denatured, and this is why porins are termed heat modifiable. This heat modification can be visualized using sodium dodecyl phosphate polyacrylamide gel electrophoresis (SDS-PAGE). Porins solubilized at room temperature will generally be seen on a gel as high molecular mass aggregates (trimers), whereas proteins solubilized at 95°C will "modify" and denature, and will be visualized as apparently lower molecular mass monomeric units (Hancock and Carey, 1979, Benz, 1985, de Cock *et al,* 1990). Another heat modifiable characteristic is that if stable monomeric subunits are isolated, samples that are solubilized at room temperature will be visualized on a gel at a lower apparent molecular mass than proteins solubilized at 95°C. This is due to the β-sheet structure of porins; the compact β-sheet protein migrates through the gel faster than an unfolded, denatured form, and heating to 95°C in SDS denatures porins from the folded to the unfolded conformation. Another attribute of porin stability is that they are normally resistant to a variety of proteases (Schulz, 1993). This characteristic is likely due to evolution; porins generally have large

surface exposed regions, and since cells may regularly be exposed to proteases, only structures that are protease resistant would evolve.

F) Model membrane studies of porin proteins

Model membrane techniques have routinely been used in the study of the structure and function of porin proteins. There are four different systems that have been most commonly used.

1) Proteoliposomes incorporating radioactive molecules (Nakae, 1975).

In this system, lipid vesicles are created using phospholipids, lipopolysaccharide, porin proteins, a ^{14}C labeled sugar that is permeable through the porin, and a ^{3}H labeled dextran which is impermeable. After the liposomes are formed, they will contain both radioisotope labels. When the solution containing the liposomes is diluted, a concentration gradient will be created, and the permeable sugar will diffuse through the porins. Liposomes can then be isolated through gel filtration, and a comparison of the retention of the permeable sugar as compared to the impermeable dextran can be measured. This assay can indicate whether or not the proteins incorporated into the liposomes actually form pores, and by using different sizes of labeled compounds, an exclusion limit can be determined.

2) Proteoliposomes containing enzymes (Tokunaga *et al.*, 1979).

This system uses liposomes that are formed from phospholipids, porin proteins, and an enzyme such as alkaline phosphatase, which becomes trapped inside the liposome. A

substrate that can be hydrolyzed by the enzyme is then added, and if it can pass through the porin protein, it will be cleaved and the amount of product that is produced can be measured. This method can give indications as to whether or not a protein functions as a porin, and it can also give some information regarding size exclusion and the kinetics of transport.

3) Liposome swelling assays (Nikaido and Rosenberg 1983).

Unlike the first two mentioned systems, liposome swelling assays do not incorporate any labeled markers into the liposomes. Instead, the swelling of the liposomes which is caused by the influx of water, causes a change in optical density which can be measured. Liposomes are reconstituted from phospholipids and porin protein in a buffer containing an impermeable solute. When liposomes are added to the test solute solution containing a permeable solute, the solute will enter the liposome according to the concentration gradient, and the osmotic gradient that is created will result in an influx of water and swelling of the liposome. The swelling causes a decrease in the refractive index of the liposome, and this decreases the optical density of the solution. This assay will, of course, give evidence as to whether or not a protein forms pores, and by using different solutes, an estimation of the exclusion limit can be made. In addition, because the log of the permeation rate is a function of the molecular size of the solute, the kinetics of uptake can be resolved, and this relationship also allows for an estimation of the channel sizes.

4) Planar lipid bilayer experiments (Benz *et al.*, 1979, Benz and Hancock

1981, Benz *et al.*, 1985).

Planar bilayer experiments involve the formation of a lipid bilayer, the insertion of porins into the bilayer, and the application of a voltage to measure the conductance of ions through the pores and across the lipid bilayer. The bilayers are typically formed across a hole connecting two compartments of a Teflon chamber. There are a number of ways of forming the bilayer and incorporating porins into the bilayer. The easiest method is by "painting" a bilayer across a hole, and then by adding porin protein (solubilized in detergent) directly to the system. The porins will eventually spontaneously insert into the bilayer. Porins can also be added in the form of proteoliposomes which will then fuse with the bilayer. Other methods utilize proteoliposome vesicles which can be used to form a monolayer on the surface of an aqueous solution. The volume of the aqueous solution can then be increased such that the level rises above that of the hole separating compartments of the chamber, and this forms a bilayer, containing porin proteins, across the hole. A schematic representation of this model system is seen in Figure 2.

When a lipid bilayer is formed, it becomes optically black to incident light, and hence the name "black lipid bilayers" by which the method is often referred. When a voltage is applied across this membrane, the lipid bilayer is virtually impermeable to the conductance of ions, but when a pore forming protein is inserted into the bilayer, an increase in conductance is observed. This system has many advantages and uses. As with the other model membrane systems, one can determine if a protein forms pores or not. By measuring

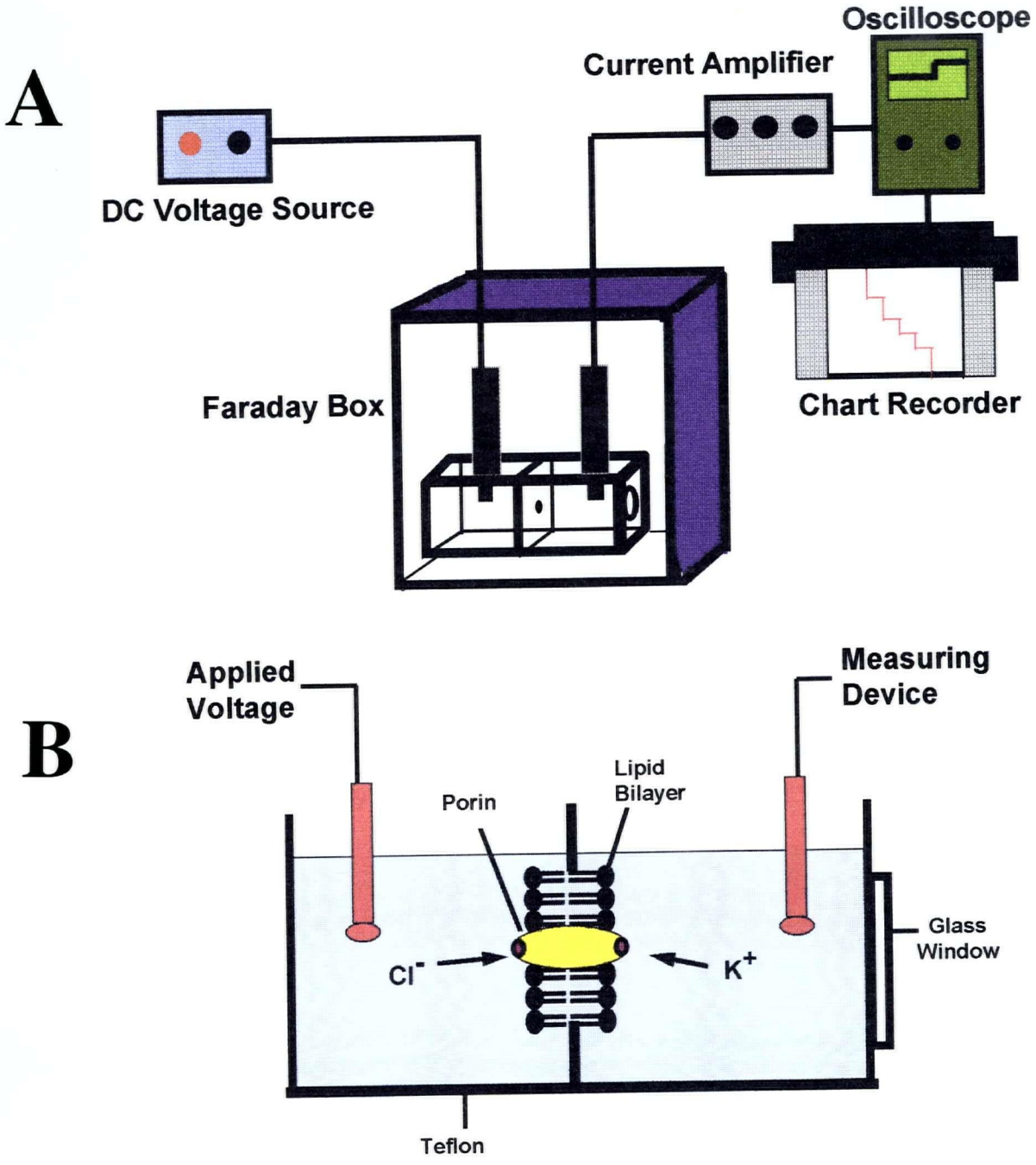

Figure 2. Schematic representation of planar lipid bilayer model membrane system. Panel A describes the experimental set up. Panel B gives a detailed representation of the Teflon chamber, and it illustrates the conductance of ions through a porin protein which has inserted into the lipid bilayer.

the conductance increases, an estimation of the channel size can also be made. Performing experiments with increasing voltages can indicate whether or not a channel is voltage gated, and zero current membrane potential experiments can indicate a porin's selectivity for anions or cations.

G) Rationale for this study

Despite the fact that porins are important to the physiology of a bacterium, nothing was known about *H. pylori* porins at the time that this study was begun. In fact, little was known about the outer membrane of *H. pylori* or about any of its membrane proteins. Various membrane proteins had been identified, but no specific functions were attributed to these proteins. There was no evidence suggesting the presence, in *H. pylori*, of classical types of highly expressed porins as one would see in the *Enterobacteriaceae*. Porins are involved in the transport of a variety of molecules across the bacterial outer membrane. More specifically, they may be involved in the uptake of molecules such as antibiotics, and thus, they may play a significant role in the apparent antibiotic resistance of *H. pylori*. Since porins are exposed on the surface of a bacterium, they can also be important in host immune stimulation. Because of the significance of porins in bacterial physiology, my studies were undertaken to identify porin proteins in the outer membrane of *H. pylori*.

The particular goals of this project were to simply identify porin proteins in *H. pylori* and to provide some characterization of the porins' functions. In doing so, it would

be necessary to devise new schemes for large scale culturing of *H. pylori* and for identifying and purifying its porins, as such methods had not been documented. By identifying and characterizing *H. pylori* porins, it was hoped that a contribution could be made to understanding the physiology of this organism, and it would also provide a basis for further study into the relationship between porins and pathogenesis in *H. pylori*.

MATERIALS AND METHODS

A) Strains, plasmids, cosmids, and phage

Helicobacter pylori CCUG 17874 (NCTC 11637) was used exclusively except where it is noted that strain CCUG 5284 was used. *Escherichia coli* strains and phage used are listed in Table II. Plasmids and cosmids used are listed in Table III. *E. coli* strains were grown on solid media composed of Luria broth and 1.5% agar. Liquid media used were Luria broth, Terrific broth, SOC medium, or TYGPN medium (Sambrook *et al.,* 1989). Strains containing plasmids were grown using the appropriate antibiotic. Ampicillin was used at 100μg/ml, and tetracycline was used at 12.5 μg/ml.

B) Growth studies

1) Viable counts and cultureability of *H. pylori*

Initial growth studies with *Helicobacter pylori* were done to determine which media would best support growth, and which would be best suited for large scale culture. The media and growth conditions used are described in Tables IV and V. For assessment of growth on solid media, cells originally grown on chocolate agar (brain heart infusion agar supplemented with 5% sheep blood cells that have been lysed by heating to 80°C for 15 min) were collected, resuspended and washed in phosphate buffered saline (PBS), and then 100 μl of the cell suspension was spread onto agar plates of varying medium compositions.

Table II. Bacterial Strains and Bacteriophage

Strain	Characteristics	Reference
H. pylori		
CCUG 17874	Type strain (*cagA*+, *vacA*+)	T.J. Trust (U. of Victoria)
CCUG 5284	Clinical Isolate	T.J. Trust (U. of Victoria)
E. coli		
DH5a	*end*A1 *hsd*R17 *sup*E44 *thi*-1 *rec*A1 *rel*A1 *gyr*A96 D(*arg*F-*lac*ZYA) U169 / f80*dlac* ZDM15	BRL, Burlington, ON
LE392	*hsd*R514 *sup*E44 *sup*58 *lac*Y1 *gal*K2 *gal*T22 *met*B1 *trp*R55	BRL, Burlington, ON
INVαF'	F' λ^- *end*A1 *hsd*R17 *sup*E44 *thi*-1 *rec*A1 *rel*A1 *gyr*A96 U169 ϕ80*dlac*ΔM15 *deo*R	Invitrogen, SanDiego,CA
Bacteriophage		
λ	λ c*Indts*857Sam7 phage	BRL, Burlington, ON

Table III. Plasmids and Cosmids

Name	Characteristics	Reference
pTZ18R	Ap^R, Multiple cloning site	Yanisch-Perron *et al.,* (1985)
pCRII	Ap^R, Multiple cloning site, predigested and containing T-overhang for efficient cloning of PCR products	Invitrogen (SanDiego CA)
pLAFR3	Cosmid with multiple cloning site, Tc^R	Staskawicz *et al.*, 1987
pHOPA	608bp *hopA* PCR product fragment cloned into EcoRI site of pCRII vector	This Study
pHOPB	110 bp *hopB* PCR product cloned into EcoRI site of pCRII vector	This study
p321	pLAFR3 clone with 20 kb *H. pylori* chromosomal DNA fragment encoding *hopA*	This study
p322	pLAFR3 clone with 20 kb *H. pylori* chromosomal DNA fragment encoding *hopA*	This study
p451	pLAFR3 clone with 20 kb *H. pylori* chromosomal DNA fragment encoding *hopB*	This study

Tc^R=tetracycline resistant, Ap^R=ampicillin resistant

Table IV. Solid growth media used

Medium *	Abbreviated name
Brain heart infusion agar	BHI
Brain heart infusion agar + 5% sheep blood (heated to 80°C)	BHI/5% blood
Brain heart infusion agar + 10% sheep blood (heated to 80°C)	BHI/10% blood
Brain heart infusion agar + 5% fetal calf serum	BHI/FCS
Brain heart infusion agar + 1% bovine serum albumin	BHI/BSA
Brain heart infusion agar + 1% casamino acids	BHI/Casamino acids
Brain heart infusion agar + 1% tryptone	BHI/Tryptone
Brain heart infusion agar + 1% skim milk powder	BHI/Milk
Brain heart infusion agar + 1% powdered hemoglobin	BHI/Hb
Brain heart infusion agar + 1% 2,6-di-O-methyl β-cyclodextrin	BHI/CD

* All media were used with or without 10 μg/ml of vancomycin, and 10 μg/ml polymyxin B (Sigma, Mississauga, ON)

Table V. Liquid growth media used.

Medium *	Growth conditions
Brain heart infusion + 5% fetal calf serum	Non-agitated growth in tissue culture flasks in 10% CO_2
Brain heart infusion + 5% fetal calf serum	Aerated growth (stirring**) in microaerobic atmosphere (Campy Pak Plus)
Brain heart infusion + 0.1% cyclodextrin***	Aerated growth (stirring**) in microaerobic atmosphere (Campy Pak Plus)

* Media were used with or without 10 μg/ml vancomycin, 10 μg/ml polymyxin B, and 2.5 μg/ml Fungizone (Gibco BRL, Burlington, ON)

** stirring was achieved by adding a magnetic stir bar and placing the sealed jar containing the culture on a magnetic stirrer.

*** cyclodextrin was (2, 6, di-O-methyl)-β-cyclodextrin (Sigma, Mississauga, ON)

The plates were incubated at 37°C in either a CO_2 incubator supplying 10% CO_2 or in sealed jars containing Campy Pak Plus pouches (Becton Dickinson, Cockeysville, MD) which supplied a microaerobic atmosphere. After 3 to 5 days of incubation, the number of colonies growing on each plate were counted, and cells from each plate were microscopically examined using wet mounts. After colony counts were made, the plates were allowed to grow for an extended period of time, and cells were subcultured at regular intervals to determine how long cells remained culturable on each medium type. Subculturing involved using an inoculating loop to obtain a sample of bacteria representative of colonies from the entire plate, and this heavy inoculum was then streaked onto another plate of the same medium composition. The presence of growth on the subcultured plate after 3 to 5 days indicated that the cells were still culturable.

Growth in liquid cultures was performed with and without shaking using the media described in Table V. Non-agitated cultures were grown in tissue culture flasks, and the culture was started by inoculating bacteria into 10 ml of medium in a 40 ml tissue culture flask. After 3 d of growth, 10 ml of fresh medium was added and after another 3 d, the culture was transferred to a 250 ml tissue culture flask, to which 30 ml of fresh medium was added. After another period of growth, 90 ml of fresh medium was added. Cells were again allowed to grow for 3 to 5 days, at which time they were added to 500 ml of fresh medium in a 2 l flask. All experiments utilizing tissue culture flasks were performed in an incubator providing 10% CO_2. Agitated cultures were grown in 30 to 60 ml of medium in 500 ml

flasks which contained a magnetic stir bar. Agitation was achieved by placing the cultures on a magnetic stirrer. Culture flasks were maintained in sealed containers, and Campy Pak gas generating systems were used to generate a microaerophilic environment. Growth in liquid culture conditions was assessed by measuring optical densities of the cultures and by counting cells using an improved Neubauer hemocytometer. The cell morphologies were also examined microscopically.

2) Culture of *H. pylori* in defined media

H. pylori cells were grown in a defined medium based on that of Reynolds and Penn (1994). The base medium (Table VI) was used along with a variety of additions or subtractions as described in Table VII. Cultures were grown in 500 ml flasks containing 60 ml of medium along with a magnetic stir bar. The flasks were then placed in a microaerophilic environment which was supplied using a sealed container containing Campy Pak Plus gas generating envelopes. The containers were then placed on magnetic stirrers, and cultures were allowed to grow at 37°C for 24 to 96 hours. Cell morphologies were examined microscopically, and all cultures were streaked onto agar plates and tested for the presence of the urease enzyme to ensure that the organisms present were *H. pylori*.

C. Protein procedures

1) Isolation of membrane proteins

Table VI. Defined growth medium for *H. pylori*

Component	Concentration (mg/l)	Component	Concentration (mg/l)
NaCl	6000	Alanine	44.5
KCl	400	Arginine	632
KNO_3	86	Asparagine	75
$MgSO_4$ $7H_2O$	100	Aspartic acid	66.5
$CaCl_2$	62	Cystine	120
Na_2HPO_4	800	Glutamic acid	73.5
$NaHCO_3$	2000	Glutamine	300
Glucose	2000	Glycine	37.5
$FeSO_4$	2	Histidine	110
Bovine Serum Albumin	5000	Isoleucine	262.5
Adenine	50	Leucine	262
Lipoic Acid	3	Lysine	362.5
D-Biotin	0.2	Methionine	75.5
Choline chloride	3	Phenylalanine	165
Folic acid	1	Proline	57.5
myo-Inositol	35	Serine	52.5
Niacinamide	1	Threonine	238
p-Aminobenzoic acid	1	Tryptophan	51
D-Pantothenic acid	1	Valine	234
Pyridoxine hydrochloride	1	Vancomycin	10
Riboflavin	0.2		
Thiamin hydrochloride	1		
Vitamin B12	0.005		
Hydrochloric acid	Variable (to bring medium to pH 7.4)		

All salt solutions, the glucose, and the HCl were obtained from Fisher Scientific, (Edmonton, AB). Adenine, and all vitamins and amino acids were obtained from Sigma (Mississauga, ON). Bovine serum albumin (fraction V) was obtained from Boehringer Mannheim (Indianapolis IN).

Table VII. Different growth media supplements or depletions

Name of Media	Deviation from Minimal Media Base
Essential A.A. deficient	0.1 X *H. pylori* essential amino acids *
Non-essential A.A. deficient	0.1 X *H. pylori* non-essential amino acids *
Essential A.A. negative	No *H. pylori* essential amino acids *
Non-essential A.A. negative	No *H. pylori* non-essential amino acids *
Essential A.A. extra	10 X *H. pylori* essential amino acids *
Non-essential A.A. extra	10 X *H. pylori* non-essential amino acids
pH 5 medium	Minimal medium at pH 5.0
pH 5 urea	Minimal medium at pH 5 with 10 mM urea
pH 7 urea	Minimal medium with 10 mM urea
$FeSO_4$ deficient	0.1 X $FeSO_4$
$FeSO_4$ extra	10 X $FeSO_4$
$FeCl_3$ supplemented	Minimal medium with 100 μM $FeCl_3$
Iron deficient	Minimal medium with 25 μM 2,2-dipyridyl
Iron depleted	Minimal medium with 250 μM 2,2, dipyridyl
Glucose deficient	Minimal medium with 0.1 X glucose
Glucose negative	Minimial medium with no glucose
Cyclodextrin +	Minimal medium without BSA and with 1g/l cyclodextrin**
CMC +	Minimal medium with 10% carboxymethyl cellulose
Copper +	Minimal medium with 1 mM $CuCl_2$
Nickel + 1mM	Minimal medium with 1 mM $NiCl_2$
Nickel + 10 mM	Minimal medium with 10 mM $NiCl_2$
Zinc + 1 mM	Minimal medium with 1 mM $ZnCl_2$
Ampicillin +	Minimal medium with 0.005 μg/ml ampicillin
Tetracycline +	Minimal medium with 0.01 μg/ml tetracycline
Metronidazole +	Minimal medium with 0.1 μg/ml metronidazole
Casamino Acid +	Minimal medium with 0.5% casamino acids
Phosphate Low	Minimal medium with 0.1 X Na_2HPO_4
Magnesium Low	Minimal medium with 0.1 X $MgCl_2$
Calcium Low	Minimal medium with 0.1 X $CaCl_2$

* *H. pylori* CCUG 17874 non-essential amino acids are: A, R, H, I, L, M, F, V (Reynolds and Penn, 1994)

** *H. pylori* CCUG 17874 essential amino acids are: D, N, C, G, E, Q, K, P, S, W, T, Y (Reynolds and Penn, 1994)

*** cyclodextrin is (2,6-di-O-methyl) -β-cyclodextrin

Large scale membrane protein isolations were performed using cells that were grown on BHI agar plates supplemented with 1% powdered hemoglobin (Accumedia, Baltimore MD). Up to 300 (150 mm x 15 mm) agar plates were streaked with *H. pylori* to form lawns of confluent growth. Cells were grown for 3 days at 37°C in an atmosphere of 10% CO_2. The cells were then harvested by scraping off the plates with a cotton swab and resuspending them in 20% (wt/vol) sucrose/10 mM Tris-HCl pH 8.0 containing 50 μg/ml of deoxyribonuclease I (DNAse) (Sigma, Mississauga, ON). The pooled cells were then disrupted by passaging the cells twice through a French Pressure Cell at 15,000 p.s.i. Any unbroken cells were then removed by centrifugation at 1000 x g for 10 minutes in a Beckman SS34 rotor. The supernatant was then layered in 10 ml quantities onto a sucrose gradient comprising (from bottom) 10 ml of 70% (wt/vol) sucrose and 20 ml of 18% (wt/vol) sucrose in a 40 ml tube, or 5 ml of 70% (wt/vol) sucrose, 10 ml of 50% (wt/vol) sucrose, and 15 ml of 18% (wt/vol) sucrose in a 40 ml tube. The samples were centrifuged at 65,000 x g for 4 h at 4°C using a Beckman SW28 rotor. The membrane bands that had migrated to a position on top of the 70% sucrose and on top of the 50% sucrose were then collected and diluted 5 fold with distilled water, and the membranes were pelleted at 250,000 x g for 1 h at 4°C in a Beckman 60Ti rotor. The membrane pellet was then resuspended in 10 mM Tris-HCl pH 8.0. Membrane preparations from liquid grown cultures were performed as above, except the cells were initially pelleted in an SS34 rotor, and washed in PBS buffer prior to resuspending in sucrose/DNAse.

Proteins were isolated from cells grown in defined media by first pelleting the cells in an SS34 rotor, and washing the cells twice with PBS. Cells were resuspended in 20% sucrose/50 mM DNAse and were then passaged once through a French pressure cell. The broken cells were diluted five fold in distilled water, and membranes were pelleted at 180,000 x g and were saved for further manipulations.

A second procedure used to isolate outer membranes was the CHAPS solubilization protocol of Worst *et al.* (1995). Cells grown in liquid media were pelleted and washed in PBS. The pellet was then resuspended in 1% (wt/vol) 3-{93-cholaidopropyl)-dimethyl-ammonio}-1-propanesulfonate (CHAPS) (Sigma, Mississauga, ON)/ 12% (wt/vol) sucrose / 50 mM sodium carbonate and was incubated at 4°C for 30 min. Following this incubation, an equal volume of 10% sodium lauryl sarcosinate (Sarkosyl) (Sigma, Mississauga, ON) was added, and the cells were incubated at room temperature for 30 min. The membrane proteins were then pelleted by centrifugation at 70,000 x g for 2h in a Beckman 70Ti rotor.

2) Assays

Protein yields were determined by using a modified Lowry assay as described by Peterson (1977). The solutions used in the assay contained sodium dodecyl sulfate (SDS), which helped to solubilize the membrane proteins.

Succinate dehydrogenase was assayed as a marker indicating the presence of inner membranes according to the protocol of Kasahara and Anraku, 1974.

Assays for ketodeoxyoctonoate (KDO) were done to determine the presence of outer membranes using a thiobarbiturate assay based on the protocol of Weissbach and Hurwitz (1958). 50 µl of 0.5 N H_2SO_4 was added to 50 µl of a membrane sample, and the mixture was heated to 100°C for 15 min. 50 µl of 2.3% periodate was then added, and the sample was vortexed, and incubated at room temperature for 10 min. 200 µl of 4% $NaAsO_2$ (in 0.5 N HCl) was added, the sample was vortexed, and then 800 µl of 0.6% thiobarbituric acid was added and the sample was heated to 100°C for 10 min. The sample was allowed to equilibrate to room temperature and then 1.5 ml of 95% butanol / 5% concentrated HCl (vol/vol) was added. The sample was vortexed and then centrifuged for 5 min at 1500 x g. The upper butanol fraction was saved, and the optical density of this fraction was measured at 549 nm and compared with KDO standards.

3) One dimensional SDS-PAGE

Protein samples were analyzed by electrophoresis through sodium dodecyl sulfate-polyacrylamide gels using a discontinuous buffer system (Laemmli, 1970). Separating gels were composed of either 11% or 15% acrylamide (29:1 acrylamide:bis acrylamide), while stacking gels were composed of 4% acrylamide. Samples were solubilized by incubation at 23°C or 95°C for 20 min in 5X sample buffer containing a final concentration of 7% glycerol and 2% SDS. After electrophoresis, the proteins were stained with Coomassie Brilliant Blue R250, or by silver (Wray *et al.*, 1981).

4) Two-dimensional (unheated x heated) SDS-PAGE

Two dimensional gels were created by first running a one-dimensional gel of a *H. pylori* membrane protein sample that was solubilized at 23°C. An entire lane was then excised from the gel, and this gel slice was wrapped in cellophane and boiled for 20 minutes in 0.375 M Tris pH 6.8. Following heating, the gel slice was fused to the top of an 11% separating gel with or without a 4% stacking gel, and electrophoresis was initiated. The gels were subsequently stained with Coomassie Brilliant Blue R250.

5) Protein purification

i) Selective protein solubilization

A series of 20 different detergent solutions (Table VIII) were tested to assess which would best solubilize the *H. pylori* heat modifiable proteins apart from other membrane proteins. Cell membrane preparations were solubilized by adding them to various detergent preparations, and the samples were then sonicated for 15 seconds. Insoluble proteins were pelleted by centrifugation at 180,000 x g, and the supernatants and pellets were analyzed by SDS-PAGE. Three detergents were ultimately chosen for use with large scale protein preparations in the following protocol. First, the membrane pellet was solubilized in 1% (vol/vol) Triton X-100 (Sigma, Mississauga, ON) /2 mM $MgCl_2$ /10 mM Tris HCl pH 8.0 by sonicating for 30 seconds, and the insoluble fraction was then pelleted at 180,000 x g for 1 hour. The pellet was then resuspended in 0.5% (wt/vol) Sarkosyl /10 mM Tris HCl pH 8.0 and the insoluble fraction was again pelleted at 180,000 x g. Finally, the pellet was resuspended in 3% (vol/vol) octylpolyoxyethylene (OPOE) (Bachem

Table VIII. List of detergent solutions tested in solubilizations of *H. pylori* membrane proteins.

Detergent and supplements
0.5 % Octyl polyoxyethylene
3.0 % Octyl polyoxyethylene
3.0 % Octyl polyoxyethylene / 0.5 M NaCl
3.0 % Octyl polyoxyethylene / 40 mM EDTA
3.0 % Octyl polyoxyethylene / 2mM $MgCl_2$
0.25% Zwittergent 10
2.5% Zwittergent 10
0.25% Zwittergent 14
2.5% Zwittergent 14
0.2 M Deoxycholate
0.05 M CHAPS
2.5% Octyl glucoside
0.08%N,N, dimethyldocecylamine-N-oxide
1.0% N,N, dimethyldocecylamine-N-oxide
1.0% Triton X-100
5.0% Triton X-100
1.0% Triton X-100 / 0.5 M NaCl
0.5% Sarkosyl
2.5% Sarkosyl
2.5% Sarkosyl / 0.5 M NaCl

Bioscience Inc., Philadelphia PA) /10 mM Tris HCl pH 8.0, the insoluble fraction was pelleted at 180,000 x g, and the supernatant was saved.

ii) Column chromatography

The OPOE soluble membrane proteins were subjected to fast protein liquid chromatography (FPLC) on either a MonoQ HR 5/5 anion exchange column (Pharmacia, Baie d'Urse, PQ), on a MonoS cation exchange column (Pharmacia, Baie d'Urse, PQ), or on a MonoP chromatofocusing column (Pharmacia, Baie d'Urse, PQ). For anion exchange chromatography, a column buffer of 10 mM Tris-HCl pH 8.0 or pH 10.0 / 0.08% or 3% N,N, dimethyldocecylamine-N-oxide (LDAO) (Fluka Chemika, Ronkonkoma, N.Y.) was used with a salt gradient of 0 to 1 M NaCl. Cation exchange chromatography utilized 50 mM acetic acid, pH 5.0 / 0.08% LDAO as a column buffer, and elution was done with a NaCl gradient of 0 to 1 M NaCl. Chromatofocusing was done using 25 mM histidine, pH 6.2 / 0.5% LDAO as the column buffer with 12.5% Polybuffer, pH 6.3 (Pharmacia, Baie d'Urse, PQ) / 0.5% LDAO as the eluent. A variety of flow speeds and sample concentrations were used in each system, and the steepness of the salt gradient was also varied. Samples were originally dialyzed into column buffer prior to loading, or in column buffer with 3% OPOE substituting for LDAO. The proteins within column fractions were analyzed by SDS-PAGE.

iii)Gel purification

After anion exchange chromatography, samples were solubilized at room temperature in SDS-PAGE loading buffer containing 2% SDS, and were electrophoresed through a preparative SDS-PAGE gel containing a 4% stacking gel and an 11% separating gel. Horizontal gel slices corresponding to the positions of the porin proteins that were visualized on Coomassie blue stained portions of the gel, rehydrated to the original size, were excised from an unstained portion of the gel. Gel slices were subsequently crushed and soaked for 24 h at 4°C in 0.5 ml of 10 mM Tris-HCl pH 8.0/1 mM ethylene diamine tetraacetate (EDTA)/150 mM NaCl/0.08% LDAO. The liquid was aspirated and filtered to remove pieces of acrylamide, and the eluate was stored at -70°C.

6) Protein sequencing

i) N-terminal amino acid sequencing

Protein samples that were partially purified by FPLC were electrophoresed on an 11% SDS-PAGE gel, and following electrophoresis, the proteins were blotted onto an Immobilon P polyvinylinedifluoride (PVDF) membrane (Millipore, Bedford, MA) using a BioRad transblot cell with a buffer composed of 192 mM glycine, 25 mM Tris pH 8.3, and 25% methanol (Millipore, Immobilon-P transfer membrane user guide). The blots were then stained with Ponceau S or Coomassie blue, and individual protein bands were excised and sent to a microsequencing facility (University of Victoria, or NAPS Unit, University of British Columbia) to be analyzed by automated Edman degradation.

ii)Internal amino acid sequencing

Protein samples were blotted using the procedure described above. After blotting onto Immobilon P^{sq} membranes (Millipore, Bedford MA) and Ponceau S staining, bands were excised and cleaved directly on the membranes as per the protocol described in Matsudaira *et al.* (1993). Briefly, 500 µl of 95% acetone was added to the PVDF slices, which were then incubated for 1h. The acetone was removed, and the PVDF slices were then placed in a formic acid/acetonitrile/cyanogen bromide solution and incubated for 24 h, at which time the supernatant was collected. The PVDF slices were rinsed with acetonitrile, the rinse solution was added to the previous supernatant, and the entire sample was dried using a vacuum dessicator, and was then resuspended in water. Following CNBr cleavage, samples were electrophoresed on a 15% acrylamide gel, and the proteins were blotted onto PVDF membranes. Protein bands were then stained with Coomassie brilliant blue R250, and visible bands were excised and sent to a microsequencing facility for sequence analysis.

D. DNA procedures

1) Plasmid procedures

i) Preparation of plasmid DNA

Small scale plasmid preparations were typically done using the alkaline lysis method (Sambrook *et al.,* 1989). Large scale preparations were carried out using a similar alkaline lysis procedure for 400 ml cultures (Sambrook *et al.,* 1989) and this was followed by CsCl density gradient centrifugation or PEG precipitation (Ausubel *et al.,* 1987). Plasmid

DNA for sequencing was isolated using a modified alkaline-lysis/PEG precipitation procedure described by Applied Biosystems Inc. (Mississauga, Ont.) in the Taq DyeDeoxy terminator cycle sequencing manual. Cosmid DNA isolations were performed in order to obtain DNA from the *H. pylori* chromosomal DNA library clones. This was done using small scale alkaline lysis, or by alkaline lysis in 96 well microtitre trays as described by Ausubel *et al.*, (1987).

ii) Transformation of plasmid DNA

Competent *E. coli* DH5a cells were prepared using a $CaCl_2$ method as described by Sambrook *et al.,* (1989), and 10 to 1000 ng of DNA was added to the competent cells for transformation (Sambrook *et al.,* 1989).

2) Isolation of *H. pylori* chromosomal DNA

H. pylori chromosomal DNA was isolated by a procedure described by Majewski *et al.* (1988). Cells were harvested from agar plates, washed, and then incubated with a 30 mg/ml lysozyme solution (in 100 mM Tris (pH 8.5) / 100 mM EDTA) for 1 h at 37°C. This was followed by a 1 h incubation after an addition of Pronase (1 mg/ml final solution) and another 1 h incubation after the addition of sodium perchlorate (0.6 M final solution). The sample was then extracted with phenol and chloroform, and the chromosomal DNA was precipitated using 95% ethanol. The DNA was subsequently pelleted and resuspended in 10 mM Tris/1 mM EDTA pH 8.0.

3) Preparation of *H. pylori* cosmid library

A cosmid library was prepared using the cosmid vector pLAFR3. *H. pylori* chromosomal DNA was partially digested with *Sau*3AI, and the DNA was centrifuged through a 10%-40% sucrose gradient for 24 h at 25,000 rpm in a Beckman SW28 rotor at 20°C. Gradients were formed with a gradient maker by using 10% and 40% sucrose solutions which each contained 1M NaCl, 5 mM EDTA, and 20 mM Tris-HCl, pH 7.5. After centrifugation, the bottom of the centrifuge tube was pierced and fractions were collected. Fractions containing DNA fragments of approximately 20 kilobase pairs (as determined by electrophoresis of samples on 1% agarose gels) were used in *in vitro* λ packaging reactions (Gibco BRL, Burlington, ON). Phage with packaged DNA were used to transfect *E. coli* LE392, and transfected cells were collected and stored at -70°C .

4) PCR cloning from the *H. pylori* chromosome

i) Oligonucleotide preparation and PCR reactions

Degenerate oligonucleotide primers for PCR reactions were created using an Applied Biosystems 392 DNA synthesizer. The oligonucleotide sequences that were chosen corresponded to reverse translated protein sequences obtained from N-terminal and internal amino acid sequences of the HopA, B, C, and D proteins, and they were designed using portions of the protein sequences that possessed the least amount of degeneracy. No codon bias was used. PCR reactions were carried out in 50 µl volumes using 0.5 µg of each 5' and 3' oligonucleotide primer, 1 µg of *H. pylori* chromosomal DNA, 2 mM of deoxynucleotide triphosphates (dNTP's), and 12 mM $MgCl_2$. Five µl of 10 X PCR buffer was used, along

with Taq polymerase (1.5 U/reaction) (Gibco BRL, Burlington, ON). Annealing temperatures were 55-56°C , and the extension time was 30 to 90 seconds at 72°C. To obtain successful results, the concentrations of primers, template DNA, dNTP's, and $MgCl_2$ were varied, as were the annealing temperatures and extension times. The above listed conditions were those which provided the best results.

ii) Cosmid library screening

The screening of the cosmid library was performed using two methods. First of all, transfected clones containing cosmid DNA from the *H. pylori* library were grown on agar plates, and colony blots were performed using nylon membranes that were positively charged by the incorporation of quaternary ammonium salts (Boehringer Mannheim, Indianapolis, IN). Blots were done according to the Genius system user's guide (Boehringer Mannheim). The blots were then probed with oligonucleotides derived from reverse translated N-terminal protein sequences from HopA, B, C, and D. The oligonucleotides were 3' end labeled with digoxigenin conjugated dideoxy uridine triphosphate (DIG-ddUTP) according to the Genius system protocol. Hybridization was done in 5X SSC buffer (750 mM NaCl, 75 mM sodium citrate, pH 7.0). Detection of positive clones was performed using alkaline phosphatase conjugated anti-DIG antibodies and Lumigen PPD (Boehringer Mannheim).

A second method of library screening involved using cosmid DNA from *E. coli* clones that had been inoculated into 96 well microtitre plates. The DNA was collected by

alkaline lysis as described and was used in a dot blot procedure in which 5 µl of each sample was spotted onto a Boehringer Mannheim positively charged nylon membrane. Prior to blotting, the DNA was denatured by subjecting it to heating or to alkaline conditions, and the DNA was then fixed to the membrane by baking at 120°C for 30 min in an oven. The membranes were probed with DNA that was randomly labeled with DIG-ddUTP. In this case, the probes consisted of a 608 bp fragment which encoded the N-terminal region of the HopA porin gene, and an 83 bp fragment encoding the N-terminus of the HopB porin gene. The fragments were obtained after PCR reactions using *H. pylori* chromosomal DNA as a template, along with oligonucleotide primers designed from N-terminal and internal amino acid sequences. PCR fragments were purified using the Geneclean II kit and protocols (BIO 101, Vista, CA). After hybridization at 68°C for 16 hours in 5X SSC buffer, membranes were washed, and detection of positive clones was performed as described in the Genius system protocol.

iii) *H. pylori* chromosome screening

H. pylori chromosomal DNA was completely digested (16h at 37°C) using various restriction endonucleases. Digested samples were electrophoresed through a 0.8% agarose gel, and were subsequently blotted onto Boehringer Mannheim positively charged membranes using an alkaline transfer procedure as described in the Boehringer Mannheim DIG labeling and detection system handbook. DNA was fixed to the membranes by baking at 120°C for 30 min, and the membranes were probed with 3' end labeled oligonucleotide

probes for HopA. B, C, and D, or with randomly labeled probes for HopA and HopB which were generated by PCR as described above.

5) DNA sequencing

Double stranded DNA sequencing was performed using an ABI 373 automated DNA sequencer. DyeDeoxy terminators (Perkin Elmer, Foster City, CA) were used in a cycle sequencing protocol which employed AmpliTaq (Perkin Elmer, Foster City, CA) as the thermal-stable polymerase. Sequencing reactions were performed according to the manufacturer's methods using DNA which was isolated using the modified alkaline lysis/PEG precipitation procedure as described in the Applied Biosystems sequencing guide.

E. Model membrane procedures

1) Single channel conductance measurements

Single channel conductance measurements were performed as previously described (Benz and Hancock, 1981). Lipid bilayers made from 1.5% (wt/vol) oxidized cholesterol in *n*-decane were formed across a 0.2 mm^2 hole connecting two compartments of a Teflon chamber containing a variety of salt solutions that were adjusted to pH 7.0 or 4.0 with 5 mM KH_2PO_4, and to pH 2.0 with 1% acetic acid. Calomel electrodes were submerged in each compartment, one connected to a voltage source, and one to a current amplifier and chart recorder (with the output monitored on a storage oscilloscope). Protein samples, solubilized in 0.1% Triton X-100, were added to one of the compartments, and a voltage of

50 mV was applied across the lipid bilayer. Conductance increases were recorded, and average conductances for each purified protein were calculated for each of the salt solutions.

2) Zero-current membrane potential measurements

Zero-current membrane potential measurements were performed as previously described (Benz *et al.*, 1985). Teflon chambers for these experiments were separated into two compartments by a divider containing a hole larger (2.0 mm^2) than those used for single channel conductance measurements. Six ml of 0.1 M KCl was added to each compartment of the chamber, a membrane was formed, and 20 ng of the porin protein was added to one of the compartments. A voltage of 20 mV was applied until the conductance reached 2.5 X 10^{-10} A, and then the voltage was removed. A 100 µl aliquot of 3 M KCl was added to one side of the membrane, while 100 µl of 0.1 M KCl was added to the other. Eight additions were made, and the zero current potential was measured after each addition.

F. Minimal inhibitory concentration determinations

Minimal inhibitory concentrations were determined by making doubling dilutions of antibiotics in 96 well round bottom microtitre trays. Each well was then inoculated with 10 µl of a *H. pylori* cell suspension. The media used was BHIB/0.1% cyclodextrin (BHI-CD) or BHI-CD with 100 µm ferric citrate and 100 µm dipyridyl, or BHI-CD with 100 µm dipyridyl. Microtitre dishes were incubated for up to 5 days at 37°C in sealed jars containing

Campy Pak Plus gas generating envelopes which provided a microaerobic atmosphere. The antibiotics used, along with their maximum concentrations were as follows: ampicillin - 0.25 μg/ml, amoxicillin - 0.50 μg/ml, tetracycline - 1.0 μg/ml, kanamycin - 2.0 μg/ml, metronidazole - 8.0 μg/ml, ciprofloxacin - 2.0 μg/ml. The minimal inhibitory concentration was judged to be the concentration which inhibited growth of cells to at least half or less (as judged visually) of the control cells, which had no antibiotics added.

RESULTS

CHAPTER 1: Formulation of *H. pylori* Growth Medium, and Optimization of Large Scale Growth Conditions

A) Introduction

At the time that these studies commenced, there were relatively few reports of the growth and growth requirements of *H. pylori*. Traditionally, to cultivate *H. pylori*, chocolate agar or blood agar was used, and these media included a rich base such as Brucella broth, or brain heart infusion broth. Liquid culture of *H. pylori* had been described (Morgan *et al.*, 1987), but growth in liquid media did not produce high cell densities. Success with liquid growth seemed to be strain dependent, and was not routinely performed in large volumes. In addition, a gyratory incubator with a gassing hood was required.

Large scale cultures were required for the purification of the *H. pylori* porins. Thus, it was necessary to identify a convenient and effective way to obtain large masses of *H. pylori* cells. This chapter describes the growth of *H. pylori* on a variety of media, and it also describes the development of large scale growth techniques, including a description of a novel and effective growth medium for *H. pylori*.

B) *H. pylori* growth studies on solid media, and identification of a novel medium formulation

Experiments were initiated to determine the growth characteristics of *H. pylori* on a variety of different solid media. Chocolate agar, consisting of 5% lysed sheep blood cells in a base of brain-heart infusion agar (BHI), was originally used to cultivate *H. pylori*. Cells grown on this media were scraped from plates, resuspended in brain-heart infusion broth (BHIB) and seeded onto agar plates containing different medium preparations. The results are described in Table IX. Growth was achieved on media containing either 1) whole blood 2) lysed blood cells, 3) fetal calf serum 4) bovine serum albumin (BSA) and 5) powdered hemoglobin (Hb). No growth was observed on media containing casamino acids, tryptone, or skim milk powder supplements.

All media which supported growth were similar with respect to the size of the colonies that were formed (1-2 mm after 5 days), the time required for individual colonies to become visible on the media (4 to 5 days), and the number of colonies that grew (Table IX). The addition to the media of antibiotics to which *H. pylori* is resistant was very helpful, since with the slow growth of this organism, it was prone to being overgrown by contaminants. Growth did not appear to be inhibited by the addition of either vancomycin, polymyxin B, or trimethoprim to the media, although when cells were plated out onto media containing all three antibiotics together, a slightly smaller number of colonies were observed. In all cases, with or without antibiotics, only approximately 2-5% of the cells which were plated actually formed colonies. This was determined by comparing the number of cells plated, as determined using a hemocytometer to the number of colonies formed.

Table IX. Growth of *H. pylori* on a variety of different media.

Media	Atmosphere	Plate Count: (10^{-5} Dilution)	CFU/ml
BHI	Microaerobic	0	0
BHI / Hb	Microaerobic	48	4.8×10^7
BHI / 5% blood	Microaerobic	56	5.6×10^7
BHI / 10% blood	Microaerobic	50	5.0×10^7
BHI / 5% FCS	Microaerobic	51	5.1×10^7
BHI / BSA	Microaerobic	49	4.9×10^7
BHI / Casamino acids	Microaerobic	0	0
BHI / Tryptone	Microaerobic	0	0
BHI / Milk	Microaerobic	0	0
BHI / Hb / vancomycin (10 μg/ml)	Microaerobic	52	5.2×10^7
BHI / Hb / polymyxin B (10 μg/ml)	Microaerobic	50	5.0×10^7
BHI / Hb /trimethoprim (10 μg/ml)	Microaerobic	48	4.8×10^7
BHI/ Hb /vancomycin /polymyxin/ trimethoprim (10 μg/ml each)	Microaerobic	40	4.0×10^7
BHI / Hb	10% CO_2	53	5.3×10^7

100 μl of cells from a culture with a density of 1×10^8 cells/ml was serially diluted and plated onto agar plates containing each medium. The plate count refers to the number of colonies observed for the particular dilution. CFU/ml is the calculated number of colony forming units per ml of cells that were plated out. Results are the average of two trials. Microaerophilic conditions were achieved using Campy Pak Plus gas generating envelopes, and the 10% CO_2 atmosphere was provided by a CO_2 incubator.

Most of the original growth studies were performed using microaerophilic growth conditions provided by activating Campy Pak Plus gas-generating packets inside sealed chambers. Because the use of these chambers was prohibitive to culturing large masses of cells using large numbers of agar plates, growth of cells in a carbon dioxide incubator was attempted. Growth in 10% CO_2 was comparable to that in a microaerophilic environment (Table IX), and it was much more convenient than growing cells in sealed chambers. Thus, further experiments were conducted comparing cells grown in microaerophilic conditions and those grown in 10% CO_2.

Cells were grown on different media and under different atmospheric conditions for up to 30 days. A comparison was then made as to which culture conditions would best support growth and maintain viable cells. Table X lists the results from these experiments. It can be seen that growth on Hb-containing medium in a 10% CO_2 atmosphere was equal to or superior to growth achieved on other media tested, and this medium and growth conditions maintained culturable cells longer than any other culture methods tested. These observations were made after examining cell morphologies by light microscopy to determine whether the cells were in the spiral/rod form, or in the non-culturable coccoid form (Table XI), or by studies observing whether or not cells could be subcultured from a particular growth medium after an increasing number of days.

In addition to maintaining viable cells for longer periods of time, the Hb containing medium was more convenient to prepare than media containing blood. Medium containing

Table X. Comparison of growth and culturability of *H. pylori* on different media and in different atmospheres.

Media	Atmosphere	Growth	Culturability
BHI / Hb	Microaerobic	++	8d
	10% CO_2	++	30d
BHI / FCS	Microaerobic	+	8d
	10% CO_2	++	10
BHI / BSA	Microaerobic	+	8d
	10% CO_2	+	8d
BHI / 5% Blood	Microaerobic	+	8d
	10% CO_2	++	16d
BHI / 10% Blood	Microaerobic	+	8d
	10% CO_2	++	16d
BHI / Casamino acids	Microaerobic	-	-
	10% CO_2	-	-
BHI / Tryptone	Microaerobic	-	-
	10% CO_2	-	-
BHI / Milk	Microaerobic	-	-
	10% CO_2	-	-

A microaerobic atmosphere was achieved using sealed jars containing Campy Pak Plus gas generating envelopes. The 10% CO_2 atmosphere was provided by a CO_2 incubator. Growth indicates the number of colonies that formed on a plate of each medium after 100 µl of a cell suspension was plated on it. Symbols: + indicates <300 colonies per plate; ++ indicates >300 colonies per plate; - signifies that there was no growth. Culturability refers to the number of days after the original plating that cells could be maintained in a culturable form; that is, subculturing produced visible growth of cells.

Table XI. Percent of cells with coccoid morphology after growth on solid media.

		Cells with coccoid morphology (%) [a]					
Media	Atmosphere	3 days	5 days	8 days	10 days	16 Days	30 days
BHI / Hb	Microaerobic	<5%	60%	80 %	>95%	>99%	100%
	10% CO_2	<5%	30%	40%	90%	>99%	>99%
BHI / 5%	Microaerobic	<5%	70%	90%	>95%	>99%	100%
	10% CO_2	<5%	50%	90%	>95%	>99%	100%

[a]The percentage refers to the approximate percentage of cells with coccoid morphology as observed in an improved Neubauer hemocytometer. Cells classified as coccoid were those showing no free ends, while all rod shapes or curved shapes were considered to be non-coccoid.

powdered hemoglobin could also be stored for at least three months and still support growth, as long as the plates were stored in sealed bags which would not allow the plates to dry out.

C) *H. pylori* growth studies in liquid media

Concurrent with the studies examining *H. pylori* growth on solid media were those examining growth in liquid media. It was known that *H. pylori* would grow in liquid media consisting of a rich base with 1 to 5% serum added (Morgan *et al.*, 1987), so studies were initiated using BHIB with 5% FCS. In addition, antibiotics were added in some cases (vancomycin, polymyxin B, trimethoprim, and fungizone). As with the solid media, virtually no differences were observed between growth with and without antibiotics. Experiments were done using non-agitated cultures grown in tissue culture flasks and in large Erlenmeyer flasks, or with agitated cultures which were grown in 500 ml flasks with stirring on a magnetic stirrer. Non-agitated cultures were started in 10 ml of BHI/FCS in 50 ml tissue culture flasks grown in a 10% CO_2 atmosphere. Fresh medium was continually added every 2 days, and cells were transferred to larger flasks as the medium volume increased. Generally, during periods after fresh medium was added, cells remained in the spiral form. As expected, coccoid cells became more abundant the longer the cells were incubated without adding fresh medium. In fresh medium, cells appeared to be extremely motile. The cells also had a definite spiral form in contrast to the more rod shaped forms seen with cultures grown on solid

media. Clumping of cells became pronounced with older cultures, but the cells appeared to become separated again upon the addition of fresh medium. Flocs also started to appear in the liquid phase of cultures grown for over three days without the addition of fresh medium. These flocs sedimented and were visually apparent, but as with the clumps, they appeared to disaggregate upon the addition of fresh medium. Cell numbers in liquid medium appeared to double in approximately 24 hours when fresh medium was added, and maximum cell densities always remained constant at approximately 1-3 x 10^9 cells/ml.

H. pylori cells grew best in shallow depths of liquid (i.e. in a tissue culture flask). When cells were inoculated into 640 ml of media in a 2 l Erlenmeyer flask, growth was very slow, and the density of the culture did not reach the levels that it did in the tissue culture flasks. In addition, cells did not appear to grow at all when inoculated directly into large volumes of liquid. It was apparent that a very large inoculum was required for any liquid growth.

Incubating *H. pylori* in 40 to 60 ml of medium in 500 ml flasks with agitation in a microaerobic atmosphere at 37°C produced some growth (10^8 cells/ml), but a large inoculum (at least 10^7 cells /ml) was required, and the maximum increase in cell numbers was only 10 to 50 fold. Agitated cultures (on magnetic stirrers) had the advantage that a larger volume of liquid and a greater depth of liquid could be used to grow *H. pylori*, and this may relate to the fact that the stirring allowed more gas exchange into the medium. Even though liquid growth could be achieved in both non-agitated and agitated cultures, the low cell densities

and the relatively small volumes of liquid that were used eliminated liquid culture as a possibility for obtaining large masses of cells. Initially, all of these liquid growth studies used BHIB supplemented with 5% fetal calf serum. However, in 1993, Olivieri *et al.* discovered that cyclodextrins could substitute for blood or serum supplements, so growth experiments were done using their proscribed media. Cells grew faster and to higher densities, but they still did not grow to high enough densities for large scale growth. Nonetheless, this media proved useful for other studies.

D) Summary

Hemoglobin supplemented medium supported growth of *H. pylori*, and using this medium it was found that maintaining the cells in an atmosphere of 10% CO_2 supported growth for an extended period of time. Hemoglobin supplemented medium maintained spiral or rod-shaped culturable cells for longer periods of time than did blood supplemented media, but there was not a tremendous difference between the different media. The addition of antibiotics to the growth media was helpful in eliminating contaminants, and vancomycin, polymyxin B, and trimethoprim could be added with little effect on the growth of *H. pylori*. Culturing *H. pylori* in liquid medium was possible, but the low cell densities which were achieved coupled with the relatively small volumes which could be used prohibited the use of liquid culture for isolating large masses of cells. The convenience of preparing the hemoglobin-containing agar medium, together with the low cost and the efficiency of this

medium for growing *H. pylori* made it the medium of choice for large scale growth of the organism.

CHAPTER 2: Identification and Purification of *H. pylori* Porin Proteins

A) Introduction

Because porin proteins are a major route for the uptake of nutrients and small hydrophilic substances in Gram-negative bacteria, and since porins have been identified in most Gram-negative bacteria, it was assumed that *H. pylori* would also have porin proteins. Due to their stable β-sheet structure and the large percentage of their surface involved in subunit-subunit interactions to form their typical trimeric structure, porins have the property of being heat modifiable. That is, they only denature after being heated in strong detergent solutions (e.g. SDS). This denaturation causes a change in the apparent molecular weights of the proteins when observed on SDS-PAGE gels, and this provides a method for detecting heat modifiable proteins, which are porin candidates.

From early studies (Dunn *et al.*, 1989), it was evident that *H. pylori* did not possess porins that were the same type as the major species that are present in most Gram-negative bacteria. In addition, the growth characteristics of *H. pylori* and the fact that the inner membrane and outer membrane of this organism did not appear to be easily separable (Goodwin and Worsley, 1993) suggested that standard procedures which were used to isolate porins from other bacteria may not be successful when working with *H. pylori*. This chapter describes the identification of porin proteins in *H. pylori*, as well as a documentation of the novel methods required to purify these proteins.

B) Preparation of *H. pylori* membrane proteins

In order to identify and purify *H. pylori* porin proteins, outer membrane protein preparations were required. Initially, membranes were obtained by growing cells on solid media, disrupting them with a French pressure cell and then isolating membranes by centrifugation through a sucrose gradient. A sucrose gradient comprised (from the bottom) of 70% sucrose, 50% sucrose, and 20% sucrose layers was originally used. Normally, the majority of the outer membranes would be found at the 50%-70% interface, while the inner membrane proteins would be at the 20%-50% interface (Hancock and Nikaido, 1978). However, when *H. pylori* membranes were isolated, this was not the case. KDO assays and SDH assays were performed to assay for the presence of outer membranes and inner membranes, respectively, as KDO indicates the presence of LPS, which is present only in the outer membrane, while SDH assays indicate the presence of succinate dehydrogenase, which is typically found in the inner membrane (Kasahara and Anraku, 1974). These assays showed that the KDO and SDH contents of both the high and low density fractions were within 10% of each other (data not shown). These results indicate that there was significant inner membrane protein contamination in the outer membrane fraction and vice versa. Polyacrylamide gels (11% SDS-PAGE) were also used to examine the protein content in the apparent inner and outer membrane preparations. Figure 3 shows the results of these gels which demonstrate that the *H. pylori* inner membrane protein samples (taken from the 20%-

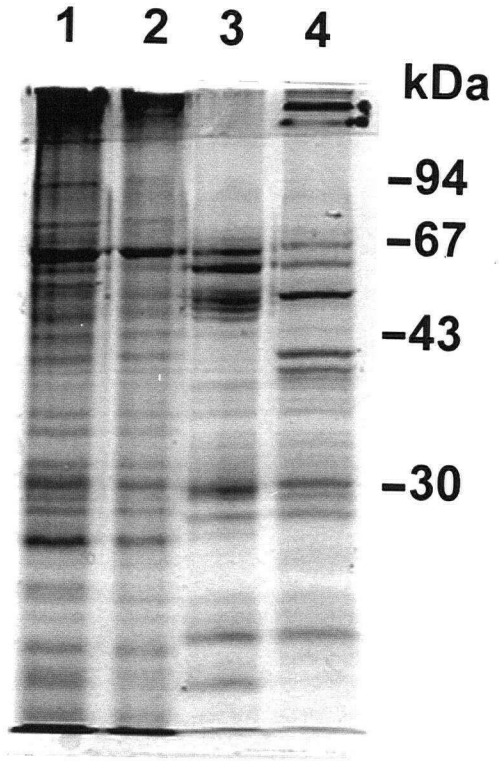

Figure 3. Coomassie Brilliant Blue stained SDS-PAGE comparison of *H. pylori* inner and outer membrane preparations. Lanes: 1) outer membrane sample solubilized at 95°C 2) outer membrane sample solubilized at 23°C 3) inner membrane sample solubilized at 95°C 4) inner membrane sample solubilized at 23°C . Membrane preparations were obtained using sucrose gradient centrifugation as described in materials and methods. Molecular masses are indicated on the right.

50% sucrose interface) and outer membrane protein samples (taken from the 50%-70% sucrose interface) gave profiles that were not identical; however, it appeared that a number of proteins with identical molecular masses were present in both inner and outer membrane fractions. The apparent presence of the same proteins in both samples along with the data obtained from KDO and succinate dehydrogenase assays suggested that the *H. pylori* inner and outer membranes were not easily separable. This is possibly not surprising, as it has not been possible to separate outer membranes from *Campylobacter* species free from inner membrane contamination (Page and Taylor, 1988). Thus, for further membrane isolations, a single step gradient (70% and 20% sucrose) was used to isolate cell envelope preparations. This ensured that no outer membrane proteins would be lost due to their partitioning into the apparent inner membrane component. Protein assays that were performed on collected membrane fractions indicated that by using cells which were scraped from approximately 300 agar plates (150 mm X 15 mm), approximately 850 mg of membrane proteins were obtained.

C) Identification of heat modifiable proteins

The property of heat-modification is characteristic of porin proteins, as they typically denature from their original trimeric organization into monomers after heating in SDS. When analyzed using SDS-PAGE, one can see the change in the porin band from a high apparent molecular mass to a lower apparent molecular mass. In addition, some porin proteins which appear to function as monomers have a different character in that the monomers increase in apparent molecular mass after heating in SDS due to the unfolding of the compact β-barrel

structure. One dimensional gel electrophoresis showed no obvious porin-like bands (Figure 3) in that there were no exceptionally predominant bands that showed heat modification from a trimeric to a monomeric configuration. However, several moderately expressed protein species appeared to change their gel mobility after the samples were heated. Because of the large number of proteins which showed a migration shift, it was not possible to determine which protein from the unheated samples corresponded to which band from the heated sample. Thus, experiments using two-dimensional SDS-PAGE (unheated x heated) were initiated.

The results of different protein separations using two-dimensional gels (data not shown) indicated that the best resolution of protein bands was obtained when using a second dimension gel without the 4% stacking gel. The protein pattern which was observed can be seen in Figure 4. At least 12 spots appeared off- diagonal and were identified as heat-modifiable proteins. There was one set of protein spots which showed gel migration patterns which would be expected from oligomers (spots at positions 1, 2, and 3 which run left of the diagonal indicating that there was a multimeric complex which had dissociated into lower molecular mass subunits after heating). However, the apparent molecular masses of two of these prospective subunits (spots at position 1 and at position 2) were similar to the molecular masses of the urease subunits (Dunn *et al.,* 1990), while the molecular mass of spot 3 was approximately 18 kDa, and was likely to be too small to be a porin (Benz, 1985, Hancock, 1986). Therefore, no further study was done on those particular proteins. The gel

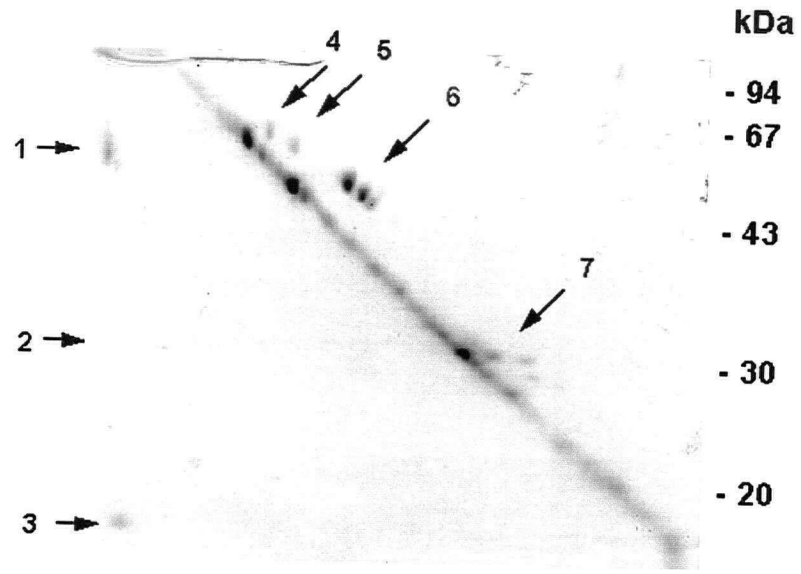

Figure 4. Identification of heat modifiable proteins by two-dimensional (unheated x heated) SDS-PAGE stained with Coomassie blue. The arrows point to spots or regions where heat modifiable proteins can be seen. Spots 1, 2, and 3 appeared to have not entered the gel without heating and are assumed to be present as poorly soluble aggregates. Spots 1 and 2 (which was barely visible), had molecular masses similar to those of urease subunits which have been shown to form high molecular weight aggregates (Dunn *et al.*, 1990) Spots designated by arrow number 6 were the original porin candidates which were to be purified. Molecular masses of standard proteins are indicated on the right.

also showed that there were a number of proteins showing migration above the diagonal, which suggested that they may be compact, stable, monomeric structures which only denatured and unfolded upon heating.

D) Purification of porin proteins

1) Selective solubilization

A detergent solubilization scheme was developed in order to selectively separate the heat modifiable proteins from contaminating proteins. Twenty different detergents and different detergent concentrations were tested to determine which would solubilize the heat modifiable proteins and which would solubilize the contaminating proteins. A group of three heat modifiable proteins were chosen as potential porin candidates. Proteins migrating with molecular masses of 37, 38, and 39 kDa before heating and 48, 49, and 50 kDa after heating were targeted, as they possessed molecular masses that were in the range observed for porin proteins (although they are at the high end of the scale), and they appeared to be fairly prominent species in the outer membrane.

After solubilizing membrane proteins in the various detergents, it was found that 1% Triton X-100 / 2 mM $MgCl_2$ / 10 mM Tris HCl pH 8.0 and 0.5% sodium lauryl sarcosinate (Sarkosyl) /10 mM Tris HCl pH 8.0 were the most effective detergents for solubilizing extraneous proteins and only solubilized small amounts of the 48, 49, and 50 kDa heat modifiable proteins. Conversely, 3% octylpolyoxyethylene (OPOE)/ 10 mM Tris HCl pH 8.0

was able to best solubilize the heat modifiable proteins with minimal solubilization of contaminating proteins. After a sequential solubilization in Triton X-100, Sarkosyl, and OPOE, analysis by SDS-PAGE showed a sample highly enriched in the heat modifiable proteins of interest (Figure 5). It could be seen that along with the 37, 38, and 39 kDa proteins (apparent molecular mass prior to heating, hereafter known as HopA, HopB, and HopC respectively), proteins with apparent molecular masses of 28, 31, 32, 33, 35, 50, 55, and 62 kDa were also present, although only the 28, 38, 39, 50, and 55 kDa proteins were prominent species. Interestingly, the 28 and 55 kDa proteins in this sample (hereafter known as HopE and HopD respectively) were heat modifiable as well, and in each case the proteins migrated with higher apparent molecular masses after heating (31 and 67 kDa respectively).

Before any further purification was performed on the OPOE solubilized sample, the sample was analyzed using a planar lipid bilayer model membrane system to examine whether or not any of the proteins in this sample actually possessed pore-forming activity. When the protein sample was added to this model membrane system, channels were observed, so it was concluded that a porin or porins were present. Thus, the proteins in the sample warranted further separation and purification to determine which specific proteins were functioning as porins.

2) Column chromatography

To separate the proteins which were enriched for in the selective solubilization procedure, Fast Protein Liquid Chromatography (FPLC) was attempted. Originally, a

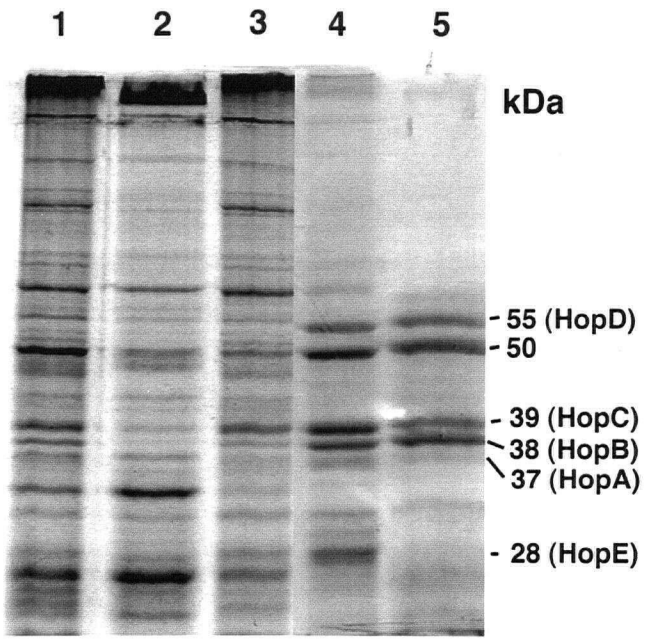

Figure 5. SDS-polyacrylamide gel of selectively solubilized, partially purified membrane proteins stained by Coomassie blue. Proteins were loaded onto gels after solubilization in SDS loading buffer at 23°C. Lanes: 1)Total membrane proteins, 2) Triton X-100 soluble membrane proteins, 3) proteins soluble in Sarkosyl after Triton X-100 extraction, 4) proteins soluble in OPOE after Sarkosyl and Triton X-100 extractions, 5)partially purified proteins isolated after anion exchange chromatography. Molecular masses are indicated on the right.

MonoQ HR 5/5 anion exchange column was used, and although it provided a separation of some of the proteins, HopB, C, and D could not be separated from each other, even after using different detergent concentrations (0.08 to 3 %) in both the sample and column buffer, or after using different salt concentrations and salt gradients. Thus, attempts were made to separate these proteins using a MonoS cation exchange column as well as a MonoP chromatofocusing column. However, as with the MonoQ column, the HopB, C and D proteins could not be separated. The protein profile of the unheated samples obtained after MonoQ anion exchange chromatography can be seen in Figure 5. A number of proteins were eliminated, and only HopB, C, D, and a protein migrating with an apparent molecular mass of 50 kDa were major species in the partially purified sample.

3) Gel purification

Since traditional techniques which had been commonly used to purify porin proteins were not successful in purifying and separating the *H. pylori* porins, preparative SDS-PAGE was used in an attempt to purify these proteins. HopB, C, and D and the 50 kDa non-heat modifiable protein were isolated by cutting protein bands out of an 11% SDS-PAGE gel. After eluting the proteins from gel slices, the resulting purified proteins were electrophoresed on an 11% gel, and the proteins were silver stained. Figure 6 shows the resulting pure proteins which were free from contaminants. The unheated samples showed faint bands that corresponded to the molecular masses of the heated samples, possibly due to the denaturation of a small percentage of the proteins during electrophoresis of the samples.

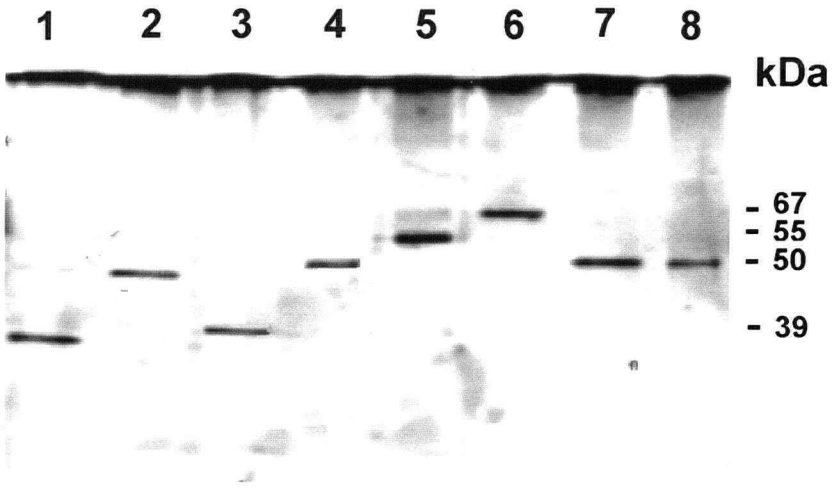

Figure 6. Silver stained SDS-polyacrylamide gel showing heat modifiability of gel purified porin proteins. Proteins were purified as described in the text with a final step of gel elution. Lanes: 1) HopB solubilized at 23 °C, 2) HopB solubilized at 95°C 3) HopC solubilized at 23 °C, 4) HopC solubilized at 95°C 5) HopD solubilized at 23 °C, 6) HopB solubilized at 95°C, 7) 50 kDa protein solubilized at 23 °C, 8) 50 kDa protein solubilized at 95°C. The HopB, C, and D proteins, with apparent molecular masses of 38, 39, and 55 kDa when solubilized at 23°C are seen with apparent molecular masses of 49, 50, and 67 kDa, respectively, when solubilized at 95°C. The 50 kDa protein was not heat modifiable. Molecular masses are indicated on the right.

E) N-terminal amino acid sequence analysis

Purified HopB, C, and D, along with the 50 kDa non-heat modifiable protein, were prepared for N-terminal amino acid sequencing. Approximately 50 pM of each sample was sequenced. The N-terminal amino acid sequences are shown in Figure 7. Even though the HopB, C, and D proteins varied considerably in apparent molecular mass, they all show strikingly similar amino acid sequences. The 50 kDa protein showed no apparent homology to the other proteins.

Also shown in Figure 7 are sequences of proteins purified by Dr. Peter Doig at the University of Victoria (Victoria, B.C.). He had purified a 48 kDa protein which migrated with an apparent molecular mass of 37 kDa prior to heating. This was similar to the molecular mass of a protein that had originally copurified with HopB, C, and D in the selective solubilization procedure and was designated HopA. It was concluded that the protein isolated by Dr. Doig was the same HopA protein. Dr. Peter Doig also isolated another heat modifiable protein which migrated with a molecular mass of 28 kDa prior to heating and 31 kDa after heating and which also possessed sequence similarity to the HopA, B, C, and D proteins, so this protein was named HopE. Interestingly, a protein with the same heat modification characteristics as HopE was also present in the sample after selective solubilizations (Figure 5).

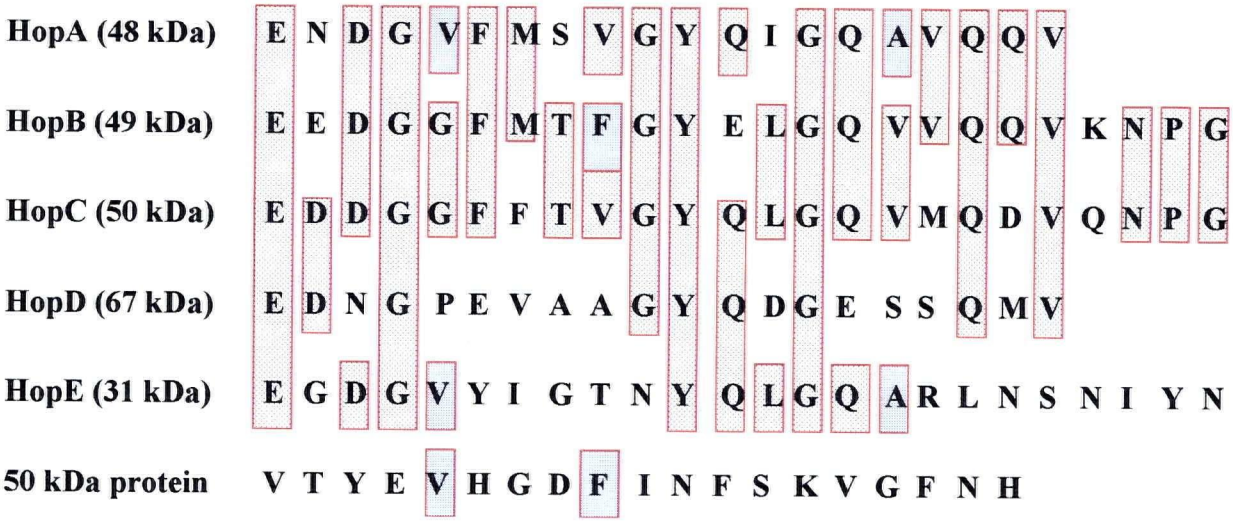

Figure 7. N-terminal amino acid sequence comparisons. Identical residues are boxed in red and shaded grey. Positions in which two different sets of proteins share homology are shaded blue. The single letter amino acid code is utilized. Accession numbers for HopA-E respectively are as follows: 957284, 957285, 957286, 957287, and 1181899.

A comparison of HopA, B, C, and D sequences with known sequences by use of the basic local alignment search tool system (BLAST) (Altschul *et al.*, 1990) showed no similarity to the sequences of other porin proteins or to any other proteins. Searches of GenPept, SWISS-PROT, and PIR data banks failed to reveal other homologs. However, HopE did show similarity to another porin protein, protein P2 from *Haemophilus influenzae* (Doig *et al.*, 1995). Residues 3 through 17 of HopE had 71% identical or conserved residues compared with the corresponding P2 porin amino acid residues. Another feature of the sequence was that an amino acid at position 28 had an aberrant retention time, indicating that this residue might in some way be modified (data not shown).

The possibility that there may be modifications to, or attached chemical constituents on these porin proteins was further suggested by an analysis of the porins from *H. pylori* CCUG strain 5284. This strain showed a different protein pattern to that of CCUG strain 17874, which was used for the protein purifications in this study, in that that the HopB and C porins from CCUG 5284 migrated on an SDS-PAGE gel with apparent molecular weights that were approximately 3 kDa greater than that of the counterparts from strain 17874 (Figure 8). An N-terminal amino acid analysis was done on the presumed HopB and C from CCUG 5284. The 23 amino acid HopB and 20 amino acid HopC sequences obtained for strain CCUG 5284 were found to be identical to the sequences (Figure 7) from strain 17874. The different mobilities of these bands were consistent with differential modifications to the

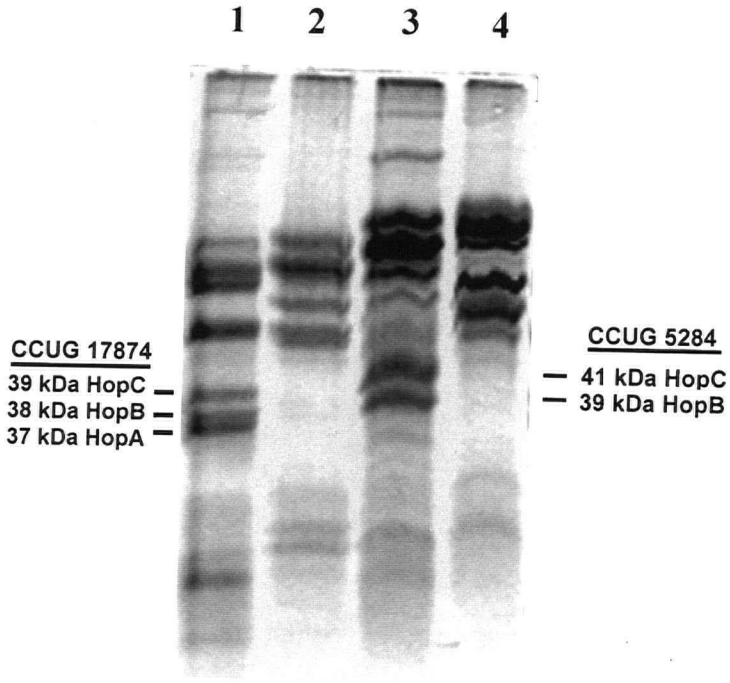

Figure 8. SDS-polyacrylamide gel of proteins from different *H. pylori* strains. Proteins solubilized in 3% OPOE after the selective solubilization protocol were loaded without heating (Lanes 1 and 3) or with heating to 95°C for 20 min (Lanes 2 and 4). Lanes 1 and 2 were proteins isolated from *H. pylori* CCUG 17874, and lanes 3 and 4 were proteins isolated from *H. pylori* strain CCUG 5284. HopA, B, and C proteins (unheated) from CCUG 17874 are labeled. Corresponding proteins from strain CCUG 5284 are visualized at slightly higher apparent molecular masses. Molecular masses are indicated on the right.

porin proteins. Alternatively, the altered apparent molecular mass could represent insertions or deletions in the porin genes of different strains.

F) Summary

Two dimensional gels showed that *H. pylori* possessed a number of heat modifiable proteins, and these proteins were considered to be porin candidates. A selective solubilization scheme designed to extract a number of these heat modifiable proteins resulted in a sample which contained 5 different heat modifiable proteins that were named HopA, B, C, D, and E. When the entire sample was added to a model membrane system, pore forming activity was observed, indicating the presence of porins, so further steps were taken to isolate and purify individual proteins. All five heat modifiable proteins were ultimately purified by elution from SDS-polyacrylamide gels (two having been obtained from Dr. Peter Doig at the University of Victoria). N-terminal amino acid sequence analysis showed that all five heat modifiable proteins possessed similar sequences, even though the apparent molecular masses differed by up to two fold. These five purified, homologous, heat modifiable proteins thus required further characterization to determine which, if any, were actually porins.

CHAPTER 3: Characterization of Porin Function

A) Introduction

The proteins which were isolated as porin candidates (Chapter 2) were all heat modifiable proteins. However, not all known heat modifiable proteins are porins. Therefore, further study was required to assess whether or not these isolated proteins were indeed porins. Various techniques are available that are able to demonstrate porin function of a particular protein, and the most utilized has been the planar lipid bilayer system (Hancock, 1986). Planar lipid bilayer systems are very versatile, as they not only indicate the pore forming ability of a particular protein, but they also enable a characterization of the equivalent size, ion-selectivity, molecular specificity (if any), and voltage gating of the porin protein (Hancock, 1986). Thus, a model membrane system utilizing planar lipid bilayers was implemented to study the potential *H. pylori* porin proteins.

The *H. pylori* proteins being examined were not present in the stable trimeric structures observed for the majority of porin proteins. Rather, they appeared to be heat modifiable monomeric units, and the studies in this chapter demonstrated this form of these proteins to have porin function. Since there was little precedent for the existence of functional monomeric porins, a number of novel potential porin proteins which appeared as monomeric units on SDS-polyacrylamide gels were obtained and studied to provide further evidence for the existence of a class of porins other than the classical trimeric porins, and to

probe the diversity of properties of such porins that function as monomers. This chapter describes the results of the experiments which demonstrated the porin function of the purified *H. pylori* proteins. This included a characterization of the proteins using a model membrane system which showed the channel forming functions of these proteins. In addition, the characterization of a number of porins from different bacteria was completed, and this showed that there appeared to be many porins, other than the *H. pylori* porins, which could function as monomers.

B) Single channel conductance measurements of HopA, B, C, D

All of the heat-modifiable *H. pylori* proteins that were isolated showed porin activity in a lipid bilayer model membrane system. Stepwise increases in conductance across the planar lipid bilayer membrane, due to the incorporation of single channels, were seen only upon the addition of purified protein in 0.1% Triton X-100 solution and not with protein-free detergent solutions alone (Figure 9). Channels were observed when as little as 1 to 2 ng of pure protein was added to a chamber containing approximately 6 ml of salt solution. Single channel conductance measurements in all salt solutions were distributed about a single mean for each of the proteins (Figure 10), and HopA, B, C, and D had similar mean conductances that ranged from 0.24 to 0.36 nS in 1.0 M KCl pH 7.0 (Table XII). In contrast, the 50 kDa protein, which was not heat modifiable, but which copurified with the porin proteins, showed

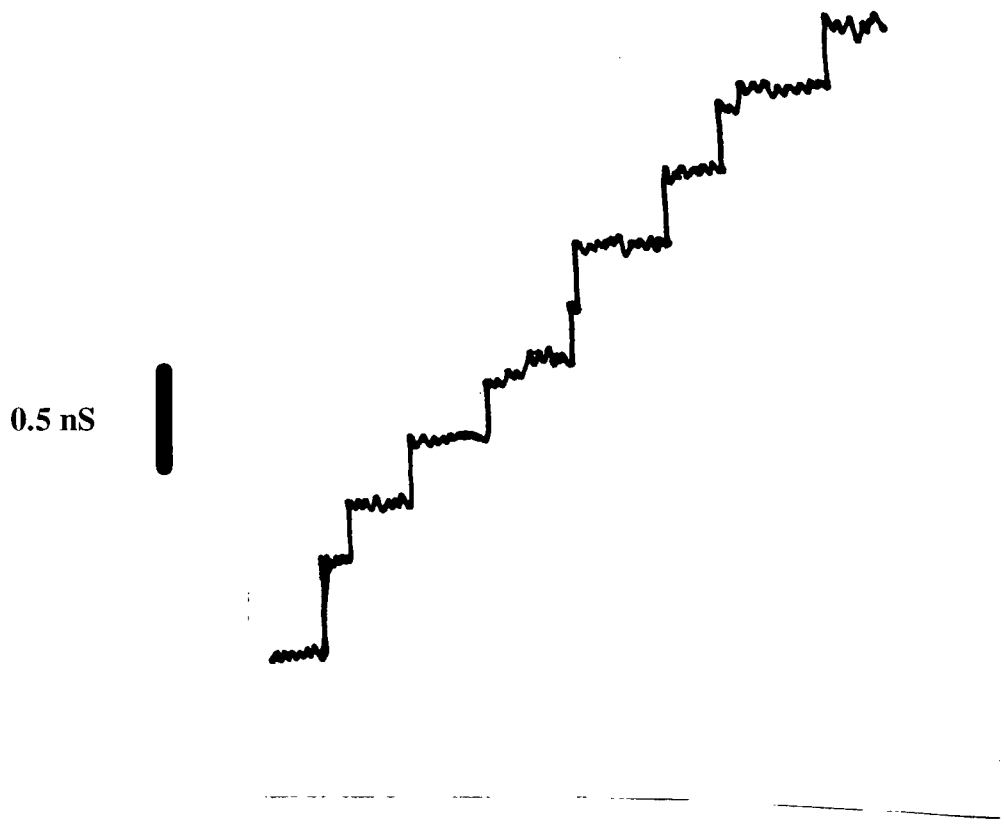

Figure 9. Stepwise conductance increases for the HopB protein. Conductance increases were recorded upon addition of 10 ng of protein to the aqueous phase (1.M KCl pH 7.0) bathing a planar lipid bilayer made from 1.5% oxidized cholesterol. The applied voltage was 50 mV. By analogy with other such studies, each stepwise increase in conductance represented the incorporation of a single pore-forming protein molecule into the bilayer.

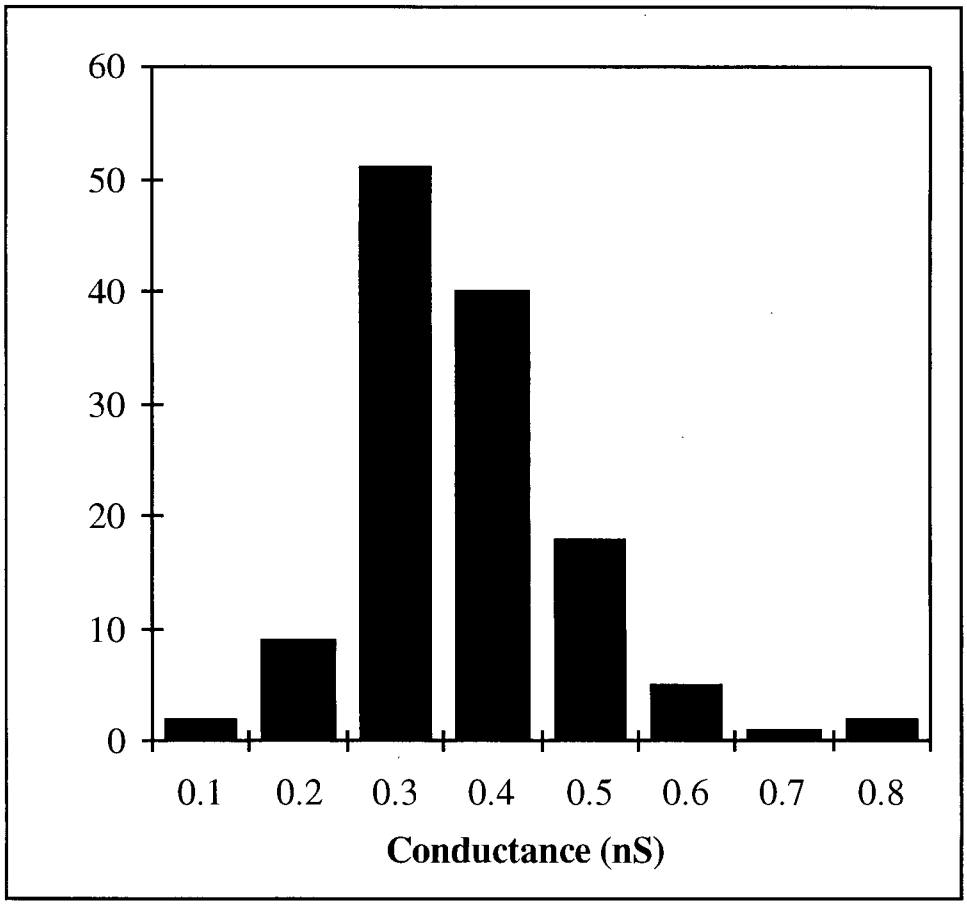

Figure 10a. Histogram of conductance increments for HopA. Porin activity assayed in a model membrane system using 1.0 M KCl pH 7.0 as the aqueous salt solution. The number of channel insertions for each channel size was charted to enable a determination of the average single channel conductance.

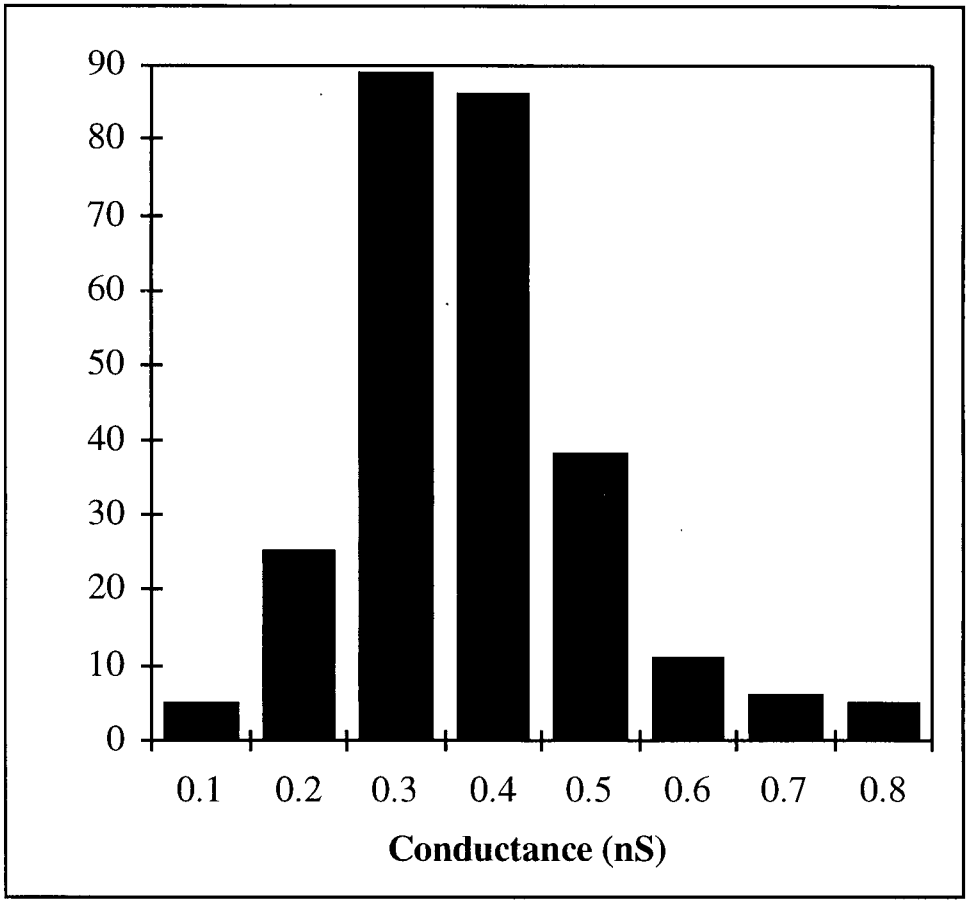

Figure 10b. Histogram of conductance increments for HopB. Porin activity assayed in a model membrane system using 1.0 M KCl pH 7.0 as the aqueous salt solution. The number of channel insertions for each channel size was charted to enable a determination of the average single channel conductance.

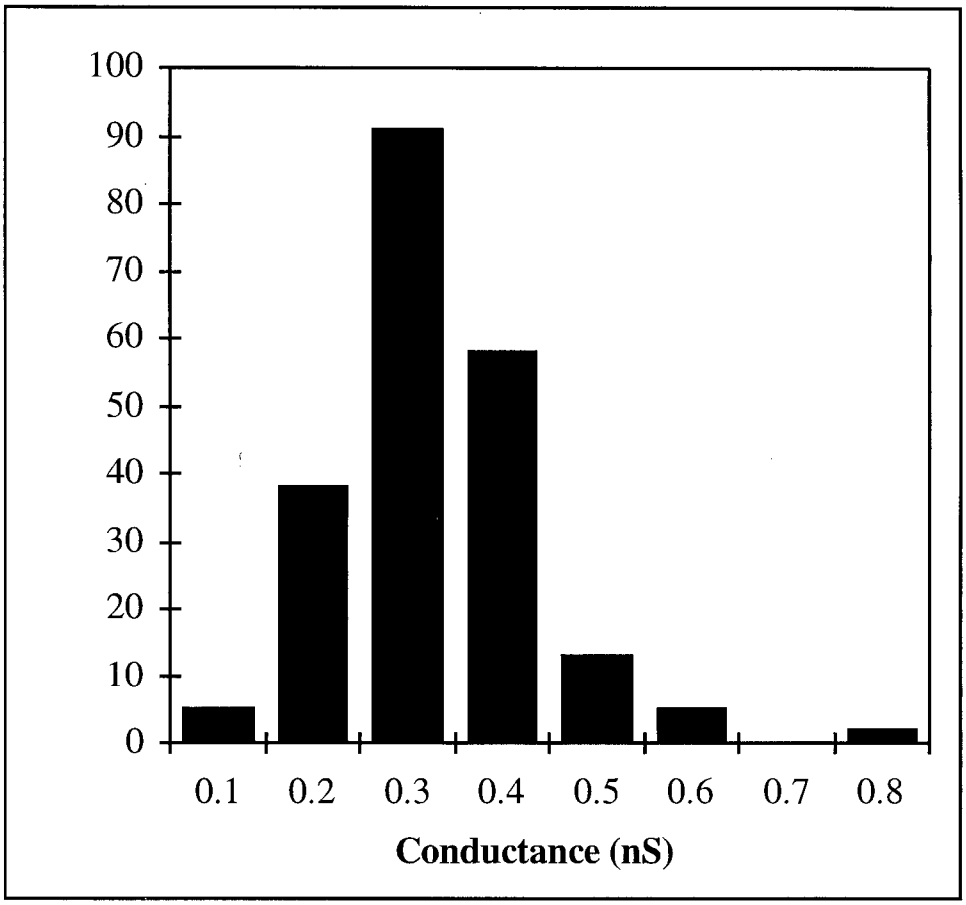

Figure 10c. Histogram of conductance increments for HopC. Porin activity assayed in a model membrane system using 1.0 M KCl pH 7.0 as the aqueous salt solution. The number of channel insertions for each channel size was charted to enable a determination of the average single channel conductance.

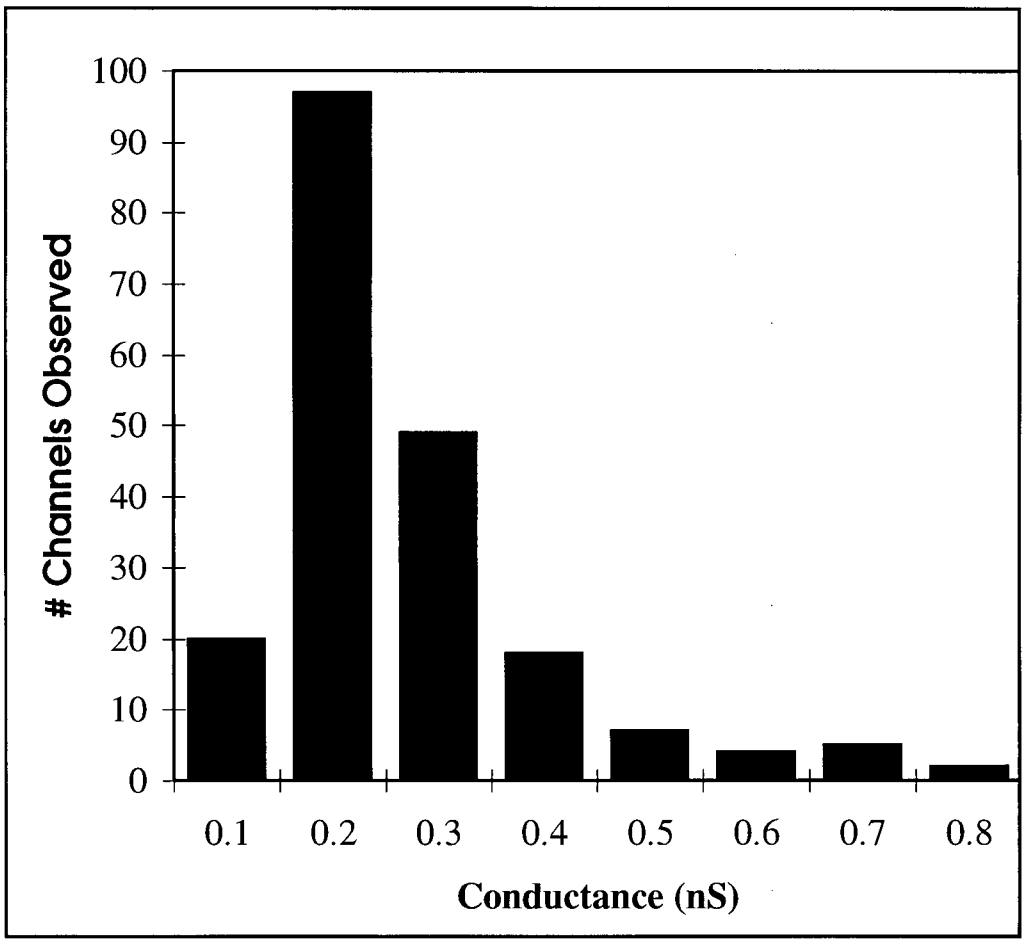

Figure 10d. Histogram of conductance increments for HopD. Porin activity assayed in a model membrane system using 1.0 M KCl pH 7.0 as the aqueous salt solution. The number of channel insertions for each channel size was charted to enable a determination of the average single channel conductance.

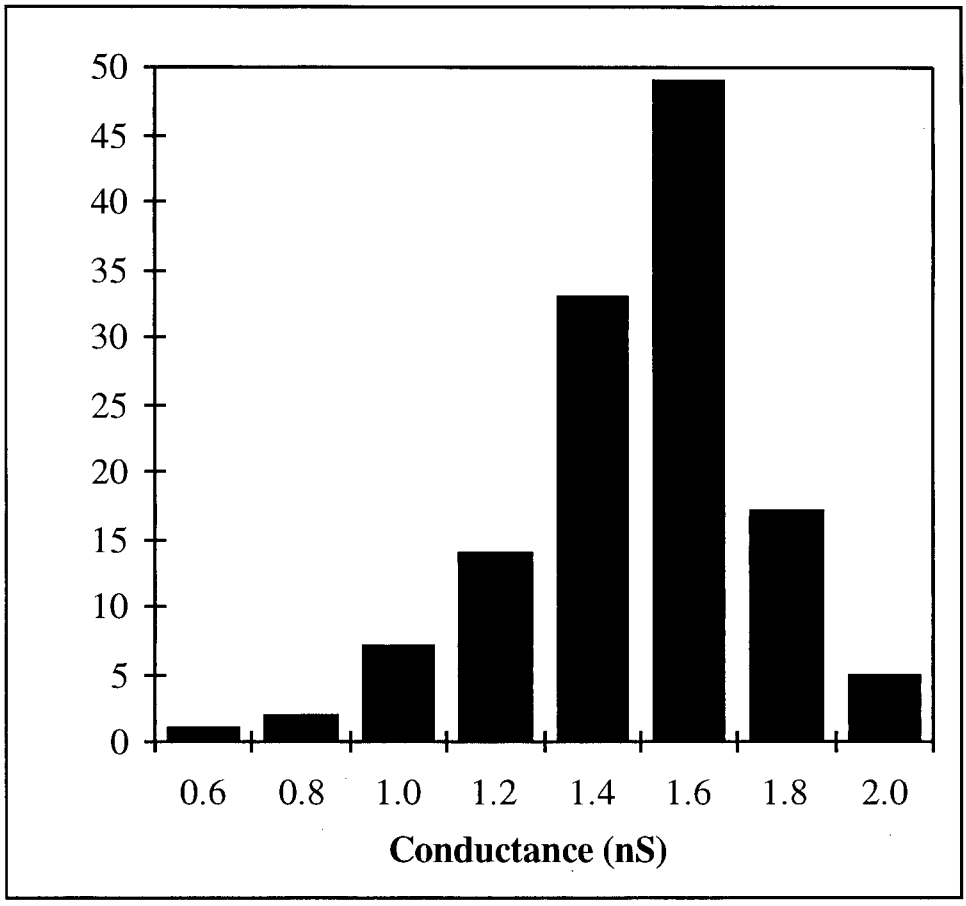

Figure 10e. Histogram of conductance increments for HopE. Porin activity assayed in a model membrane system using 1.0 M KCl pH 7.0 as the aqueous salt solution. The number of channel insertions for each channel size was charted to enable a determination of the average single channel conductance.

Table XII. Average single channel conductance measurements for *H. pylori* porin proteins in a variety of salt solutions.

	HopA		HopB		HopC		HopD		HopE	
Salt	G (nS)	n	G (nS)	n	G (nS)	n	G (nS)	n	G (nS)	n
0.3 M KCl			0.12	98	0.11	105	0.09	172	0.45	88
1.0 M KCl pH 7.0	0.36	128	0.36	265	0.32	210	0.24	198	1.50	129
1.0 M KCl pH 4.0			0.23	106	0.31	127	0.25	149		
1.0 M KCl pH 2.0			0.10	66	0.20	42	0.24	36		
3.0 M KCl			0.68	104	0.64	194	0.66	182	2.95	90
1.0 M LiCl			0.20	97	0.24	158	0.27	128	0.86	89
1.0 M KCH_3COO			0.11	90	0.13	158	0.09	136	0.65	103

G was the average single channel conductance. All measurements are in nS. HopA was only tested in 1.0 M KCl as the amount of sample was limited. n represents the number of channels observed. The pH of the salt solutions was 7.0 unless otherwise noted.

no pore forming activity. The low conductances observed in all cases with KCH_3COO as the mobile salt, compared to those for LiCl (Table XII), were consistent with a weak selectivity for anions over cations since K^+ and Cl^- are much smaller than hydrated Li^+ and CH_3COO^- respectively. HopB and HopC showed marked decreases in single channel conductance at lower pH values, while HopD appeared to be unaffected (Table XII). HopB channels changed from a conductance of 0.36 nS at pH 7.0 to a conductance of 0.23 at pH 4.0, and a conductance of 0.10 at pH 2.0. HopC channels showed virtually the same conductance at pH 7.0 and pH 4.0 (0.32 and 0.31 nS respectively), but the conductance dropped to 0.20 nS at pH 2.0. HopA was tested in 1.0 M KCl pH 7.0 and not in other salt solutions because only a limited amount of purified protein was available.

C) Single channel and zero current membrane potential measurements of HopE

Purified HopE exhibited pore-forming ability in a planar lipid bilayer system upon the addition of approximately 10 ng of protein to the solution bathing a membrane composed of 1.5% oxidized cholesterol. Similar to the other Hop proteins, the single channel conductances observed for each salt solution were distributed about a single mean, and the conductances were found to be a linear function of the salt concentration. The average single channel conductance for the monomeric units was 1.5 nS, which was considerably larger than the conductance of HopA, B, C, and D. Zero current membrane potential measurements were taken to determine the ion selectivity (if any) of HopE. The ratio Pc/Pa for HopE, where Pc

is the permeability for cations and Pa is the permeability of anions, was calculated by the Goldman-Hodgkin-Katz equation to be 1.059 ± 0.001, which indicated that the protein showed virtually no selectivity for cations or anions. This was consistent with the observation that there were similar decreases in conductance when using either a larger cation (Li^+) or a larger anion (CH_3COO^-) (Table XII).

D) Model membrane analysis of functional monomeric porins from other bacteria

Contrary to observations that nearly all porins that had been previously isolated were functional as trimeric units, all of the *H. pylori* porins that were studied here were apparently functional *in vitro* as monomers [although there is evidence that HopE may be associated in trimers (Doig *et al.,* 1995)]. To further investigate the possibility that porins functional as monomers may be more widespread that previously thought, a number of porin proteins from a variety of organisms, including 3 spirochetes (Blanco *et al.,* 1995, Gherardini *et al.*, unpublished results, Lutwyche *et al.*, 1995 Shang *et al.*, 1995, Forst *et al.*, 1996), were examined to determine whether or not they could function as monomers .

These porins were purified by others by using, as a final step, the gel purification and elution procedure which I had developed for purifying the *H. pylori* porins. The porin proteins and the organisms that they were obtained from are listed in Table XIII. As can be seen, there were a large variety of organisms from which monomers, that I was subsequently able to demonstrate to be functional as porins, were isolated. There was significant variation

Table XIII. Characteristics and single channel conductances of porin proteins that function as monomers.

Organism / Protein	Monomer Mol. Wt. (kDA)	Evidence of Multimer	Conductance (nS) of Monomer in 1.0 M ICl	Reference
Treponema pallidum Tromp1	31	yes	0.70	Blanco *et al.,* 1995, this thesis
Treponema pallidum Tromp2	28	no	0.30	Champion *et al.,* 1996, this thesis
Borrelia burgdorferi 39 kDa	39	yes	0.56	Gherardini *et al.,* unpublished;this thesis
Leptospira kirschneri OmpL1	31	yes	1.10	Shang *et al.,* 1995; this thesis
Aeromonas hydrophila 28 kDa	33	no	1.96	Lutwyche *et al.,* 1995;this thesis
Xenorhabdus nematophilus OpnP	30	no	0.44	Forst *et al.,* 1995; this thesis
Helicobacter pylori HopA	48	no	0.36	Exner *et al.*, 1995, this thesis
Helicobacter pylori HopB	49	no	0.36	Exner *et al.*, 1995, this thesis
Helicobacter pylori HopC	50	no	0.32	Exner *et al.*, 1995, this thesis
Helicobacter pylori HopD	67	no	0.24	Exner *et al.*, 1995, this thesis
Helicobacter pylori HopE	31	yes	1.50	Doig *et al.*, 1995, this thesis

in the conductance and therefore the size of these channels. A more thorough analysis of each of these channels was performed by me and is included in the references listed in Table XIII.

E) Summary

Five different heat modifiable proteins which were isolated and purified from *H. pylori* have been shown to function as porins. HopA, B, C, and D showed very similar pore forming characteristics; they appeared to function as monomers, and formed pores with single channel conductances in 1.0 M KCl ranging from 0.24 to 0.36 nS. Two of the proteins (HopB and HopC) showed marked decreases in conductance at lower pH values. These four porins appeared to have a slight selectivity for anions over cations. HopE showed distinct pore forming characteristics from the other four porins, as it formed significantly larger channels with an average conductance of 1.5 nS. Zero current membrane potential measurements demonstrated that this porin showed virtually no selectivity for anions or cations.

Since all five *H. pylori* porins were functional as monomers, they differed from the majority of porins isolated and characterized to date. Thus, studies were undertaken to examine whether or not monomeric functional porins could be isolated from other species. Six different monomeric porins from 5 different genera of bacteria were isolated by different laboratories and shown to be functional in these thesis studies. This showed that the *H. pylori* porins and previously described monomeric porins were not necessarily unique in their

structure.

CHAPTER 4: Growth of *H. pylori* in defined media, and examination of porin protein expression

After it had been shown that HopA, B, C, D, and E functioned as porins, studies were performed in hopes of elucidating whether or not there were specific functions for these porin proteins other than being general diffusion pores. The approach used was to grow *H. pylori* in defined media with various supplements and monitor porin protein expression. It was hoped that some differential expression of the porins would be observed under different growth conditions or in different media, and this would suggest specific roles for the porins.

Prior to growing cells in defined media, experiments were undertaken to determine whether porin expression differed for cells grown on BHI supplemented with blood, hemoglobin, or cyclodextrin, and additionally, cells were grown for different lengths of time and in different atmospheres to see if those conditions altered expression. After isolating cells that had been grown in the various conditions, whole cell lysates, membrane preparations, and selectively solubilized proteins were all examined using SDS-PAGE. Protein profiles and levels of protein expression from cells grown on the different media were compared and analyzed. The initial results showed that there were minimal differences in protein expression with all of the solid media used (Figure 11). Growth in BHI/CD liquid medium

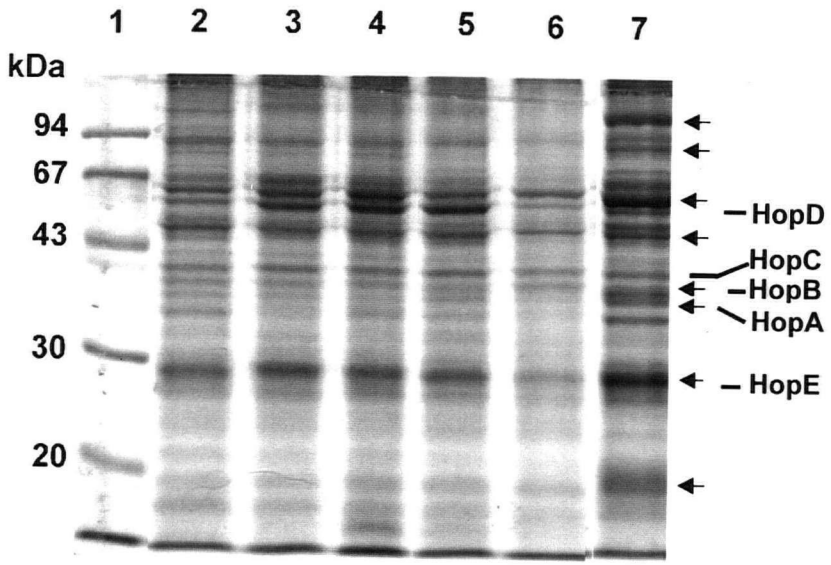

Figure 11. SDS-PAGE of membrane preparations from cells grown on different media and for different periods of time. Lane 1 was a series of molecular weight standard proteins. Lane 2 was from cells grown on BHI/5% blood for 3 d in a microaerophilic environment, lanes 3 and 4 were from cells grown for 3 d and 14 d respectively on BHI/5% blood in 10% CO_2, lanes 5 and 6 were from cells grown for 3d and 30d respectively on BHI/Hb in 10% CO_2, and lane 7 was from cells grown 3d in BHI/CD liquid medium in a microaerobic atmosphere. Molecular masses of standard proteins are indicated on the left, and proteins with increased expression in BHI/CD are indicated with arrows. The positions of the *H. pylori* porins is marked on the right.

(Figure 11, lane 7) resulted in an increase in the expression of HopA and B as compared to growth on the different solid media, along with increases in expression of proteins with apparent molecular masses of 100, 90, 65, 46, 28 and 19 kDa. The number of times a strain was subcultured and the atmosphere in which it was grown did not appear to affect porin expression. The length of time cells were incubated in culture only resulted in altered protein profiles when cells were grown for 30 days [cells grown 3 days and 14 days showed the same protein profiles (Figure 11, lanes 3 and 4)]. Cells grown for 30d had lower expression levels for virtually all of the proteins in the sample. Experiments were then continued using defined liquid media with different supplements (Tables VI and VII in materials and methods). No major increases or decreases in porin expression were seen. However, growth with different iron concentrations appeared to result in some alterations in expression of proteins with molecular masses corresponding to HopA, B, and C. Thus, growth with varying iron concentrations was examined in further detail.

Continuing experiments using iron depleted medium or medium with excess iron showed that expression of HopA, B, and C was definitely influenced by the iron concentration in the medium. The outer membrane CHAPS solubilization procedure outlined by Worst *et al.* (1995) was used to isolate membrane proteins from cells grown in BHI-CD with different concentrations of 2,2-dipyridyl, which was used to chelate free iron. The use of chelators to create iron limitation is standard and is more effective than utilizing additions of different amounts of iron, as the iron present as a contaminant in medium supplements or

bound to the sides of growth containers may lead to improper calculations of iron concentration. Limiting the iron concentration in the defined media by using chelators resulted in low cell yields. Since BHI/CD media gave better cell yields than the defined media, BHI/CD was used for experiments using chelators. Membrane preparations derived from cells grown in iron depleted conditions (BHI-CD with 250 μM dipyridyl) showed increased expression of HopA, B, and C. In addition, 28, 36, and 50 kDa non-heat modifiable proteins appeared to be increased in expression levels relative to those under conditions of iron sufficiency (Figure 12, *cf* lanes 3,4 and 1,2). When less dipyridyl was added (25 μm) resulting in a more modest iron deficiency, there was still an increase in the expression of the previously mentioned proteins, but to a lesser extent.

After finding that the expression of certain *H. pylori* porin proteins was iron regulated, experiments were performed to determine whether porin expression levels had an effect on antibiotic susceptibility. It is known that some antibiotics use porin channels to enter a cell, and it has been proposed that pore forming proteins [e.g. OprM from *Pseudomonas aeruginosa* (Poole *et al.,* 1993)] are involved in the efflux of antibiotics out of the cell. Thus, minimal inhibitory concentration assays (MIC's) were performed using a variety of antibiotics with cells grown under conditions that influenced porin expression. The results (Table XIV) indicated small but consistent decreases in MIC's for the β-lactam antibiotics ampicillin and amoxicillin under conditions favorable to porin overexpression. In contrast, these same conditions rendered the cells less susceptible to tetracycline and ciprofloxacin.

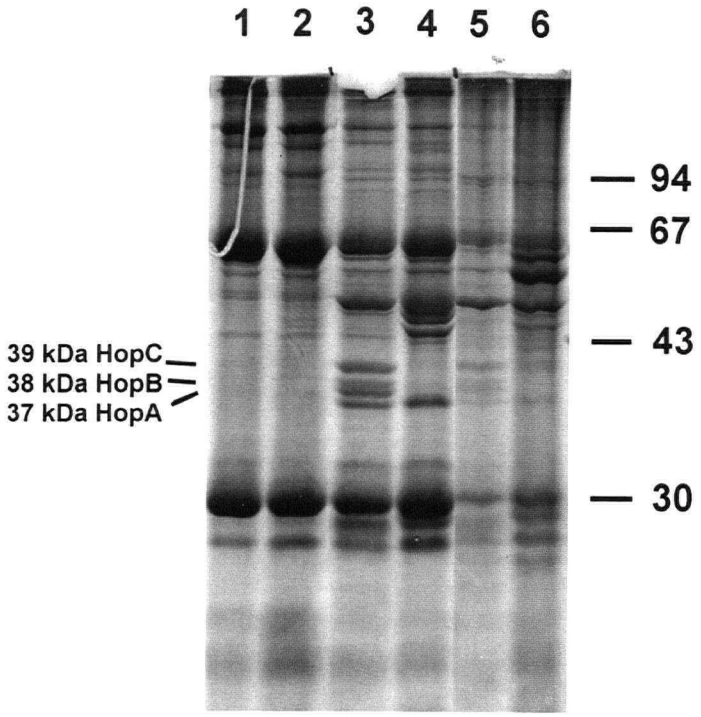

Figure 12. SDS-PAGE showing regulation of porin expression by iron. Samples grown in different media were solubilized using the CHAPS solubilization procedure (Worst *et al.*, 1995). Samples were obtained from cells grown in BHI-CD supplemented with 100 μm $FeCl_3$ (lanes 1 and 2), 250 μm 2,2-dipyridyl (lanes 3 and 4), or 25 μm 2,2-dipyridyl (lanes 5 and 6). Samples from lanes 1, 3 and 5 were solubilized at 23°C, while those from lanes 2, 4, and 6 were solubilized at 95°C. The positions for HopA, B, and C are seen on the left, and molecular masses are indicated on the right.

Table XIV. Minimal inhibitory concentrations for *H. pylori* grown with varying iron concentrations

Media	Ampicillin	Amoxicillin	Tetracycline	Kanamycin	Metronidazol	Ciprofloxaci
BHI-CD	0.25	0.016	0.06	0.50	1.0	0.06
Fe Deficient+Fe	0.12	0.016	0.06	0.50	0.50	0.06
Fe Deficient	0.06	0.008	0.25	0.50	1.0	0.13

BHI-CD is brain heart infusion broth supplemented with 0.1% cyclodextrin. Fe Deficient + Fe is BHI-CD + 100 μm dipyridyl + 100 μm Fe Citrate. Fe Deficient is BHI-CD with 100 μm dipyridyl. All MIC values are in μg/ml.

The results from this chapter showed that some porin expression levels were increased under conditions of iron deprivation. Of the porin proteins, only HopA, B, and C appeared to be affected, but other proteins also showed increased expression under these same conditions. In addition, it was found that antibiotic susceptibility changes occurred coincidentally with these changes in protein expression. Those antibiotics which typically traverse porin channels [i.e. β-lactam antibiotics (Hancock and Bell, 1988, Livermore, 1988)] showed increases in susceptibility with higher levels of porin expression, while antibiotics for which bacteria have active efflux systems [i.e. tetracycline and ciprofloxacin (Hancock and Bell, 1988)] showed decreases in susceptibility with increased porin expression.

CHAPTER 5: Cloning and DNA Sequence of Porin Proteins

A) Introduction

Once the 5 *H. pylori* porin proteins had been isolated and characterized with respect to pore-forming ability, steps were taken to clone one or more of the porin genes. The primary purpose for cloning these genes was simply to analyze the sequences and to determine if the similarity between the five *H. pylori* porins extended beyond the N-terminal region. It would be interesting to map the porin genes to the *H. pylori* chromosome, and to discover whether or not the apparently related proteins are co-localized on the chromosome. In general, cloning of the genes would also allow for a multitude of further manipulations of the genes and proteins, and as a short term goal, the sequencing and localization of the genes on the chromosome would provide further information regarding porin structure and whether or not the porins were found in a regulon. This chapter describes the construction of a *H. pylori* gene bank, the identification of cosmid clones which encoded *H. pylori* HopA and HopB, and the hybridization of these clones to *H. pylori* chromosomal DNA.

B) Construction of *H. pylori* gene bank

A gene bank of *H. pylori* chromosomal DNA was created by partially digesting chromosomal DNA with *Sau*3AI and ligating 20 kilobase pair size fractionated fragments into the cosmid vector pLAFR3. The resulting cosmids were packaged into λ phage heads,

and were then transfected into *E. coli* LE392, plated out, and ampicillin resistant transfectants were collected. It was determined that the library consisted of 5 x 10^8 colony forming units per ml. Alkaline lysis minipreps were made using 30 randomly selected clones. The isolated cosmid DNA was then digested with EcoRI and with EcoRI /HindIII. Because all of the clones showed different digest patterns, it was assumed that the library was likely to be representative of the entire chromosome, as no specific chromosomal DNA fragments predominated.

C) Construction of porin gene probes and DNA sequencing of probes

To identify the porin genes in the cosmid library, probes for the porin genes were required. Originally, the only sequences available which could be used as probes were the degenerate reverse translations of the N-terminal amino acid sequences. Degenerate oligonucleotide probes were created using these sequences, and the oligonucleotides were then 3' end labeled using digoxigenin labeled dUTP (DIG-dUTP) according to the Boehringer Mannheim 3' end labeling kit protocol. The sequences used as probes for *hopA, B, C*, and *D* can be seen in Figure 13. These probes were used to screen colony blots and dot blots of cosmid DNA that had been isolated from a number of clones, but no positive clones were found, possibly due to the low sensitivity of the 3' end labeling and because only small amounts of DNA were present in each colony being examined.

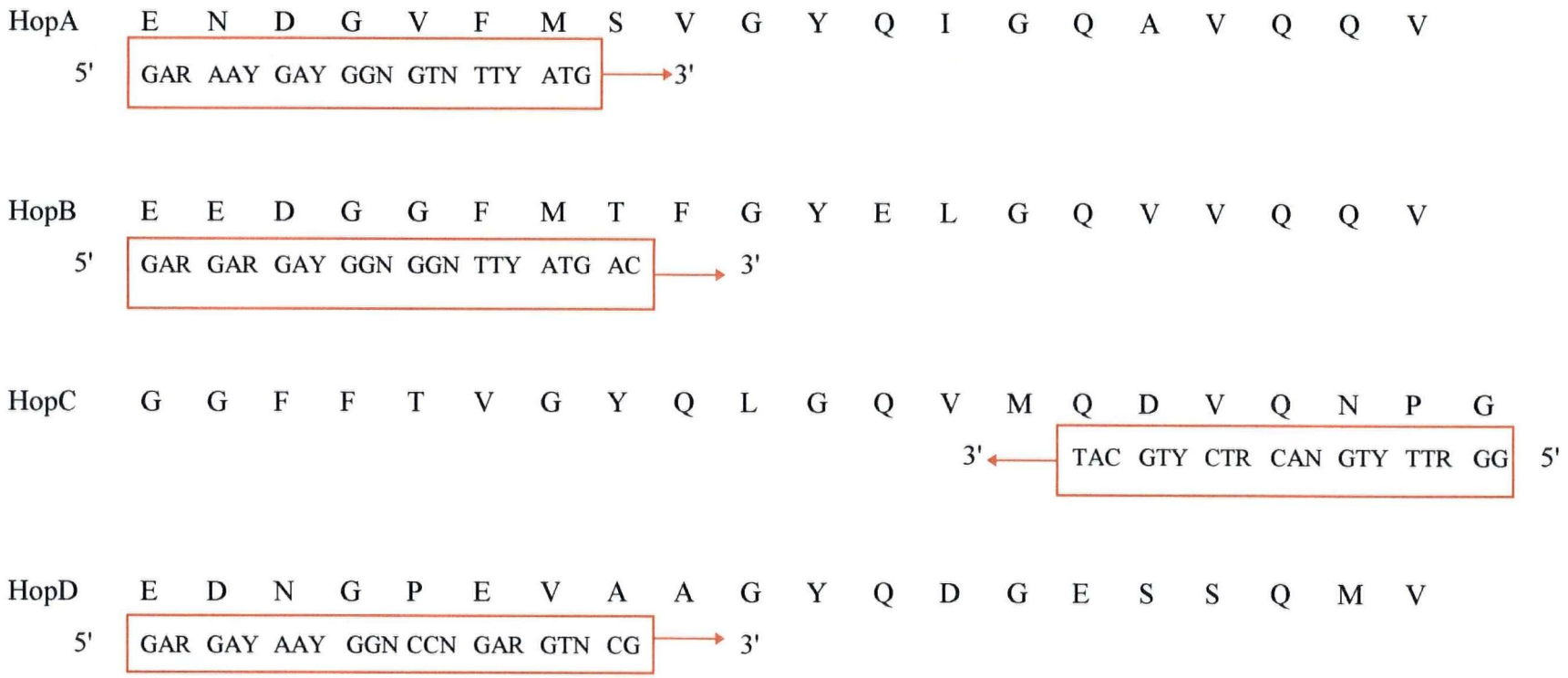

Figure 13. Oligonucleotides used for porin probes. The partial N-terminal amino acid protein sequences are presented in the single letter code, and reverse translated nucleotide sequences are boxed in red. For nucleotides, R =A or G, Y=C or T, and N=A or C or G or T

Since these methods proved unsuccessful, a new scheme to provide probes was developed. HopA, B, C, and D were purified, and the proteins were cleaved with CNBr, which cleaves after methionine residues (Lehninger, 1970). The cleavage pattern for these proteins is seen in Figure 14, and it shows that even though the proteins showed molecular mass differences of up to 19 kDa, protein bands with apparent molecular masses of 8, 10, 13, and 17 kDa were seen in corresponding positions in more than one sample. Cleavage products were isolated and sent to a protein micro-sequencing facility for N-terminal amino acid sequencing. Internal sequences were obtained for all four proteins, and the sequences can be seen in Figure 15. Sequences for HopA and HopC were obtained from 10 kDa peptides, and sequence alignment shows that there was some minimal similarities in these internal sequences. The sequence from HopB was from an 8 kDa peptide and the HopD sequence was obtained from an 11 kDa peptide, and the sequences again showed some slight similarity. When these proteins were aligned with HopA or C, no obvious similarity was detected. Using N-terminal and internal protein sequences, PCR primers were created such that the region from the N-terminus to the point at which the internal sequence was located could be amplified. An outline of the strategy used to create PCR products which encode portions of the porin genes is seen in Figure 16. The oligonucleotide primers that were used are shown in Figure 17. A large number of PCR reactions were performed, utilizing different annealing temperatures, different extension times, different templates, and different, $MgCl_2$, dNTP, template, and primer concentrations. In all cases, controls using no template were

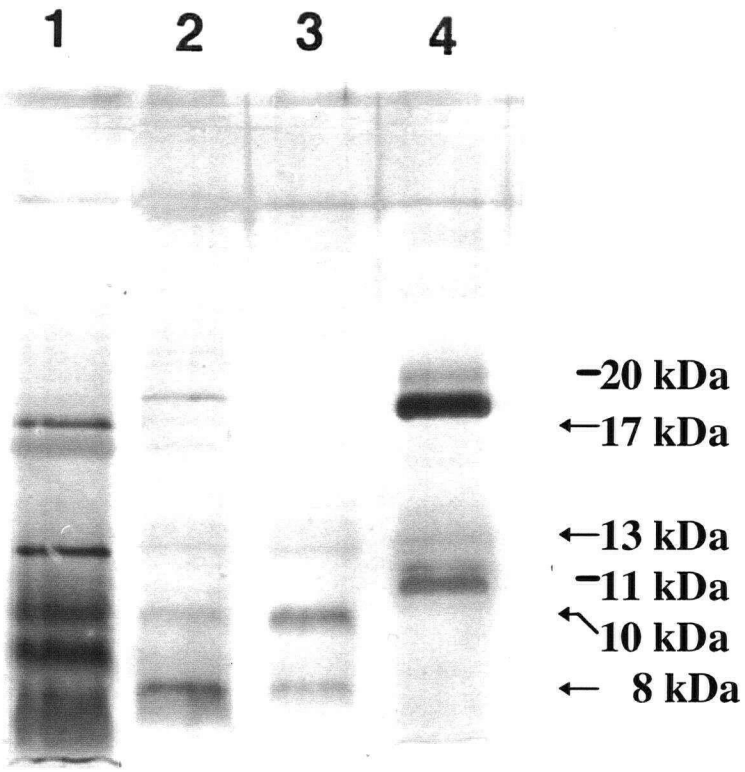

Figure 14. Silver stained 15% SDS-polyacrylamide gel of peptide fragments generated by CNBr cleavage of HopA, B, C, and D. Lanes: 1) HopA; 2) HopB; 3) HopC; 4) HopD. Protein fragments were solubilized at 95_C for 20 min prior to loading. Molecular masses are indicated on the right. Positions where bands of similar molecular masses are seen in multiple lanes are indicated with arrows.

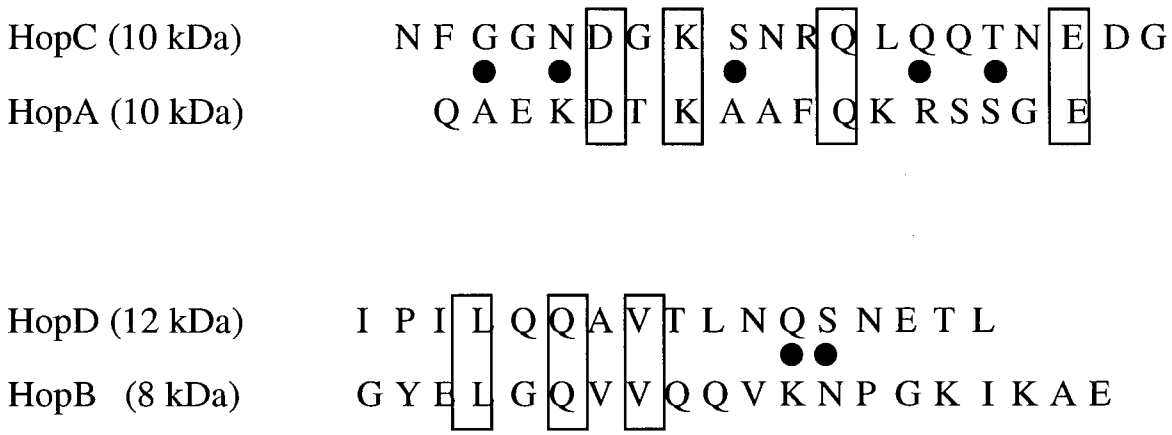

Figure 15. Internal amino acid sequences of CNBr digested HopA, B, C, and D peptide fragments. Identical amino acids are boxed, and "●" designates similar amino acids [using a cut off score = 9 with the Dayhoff similarity matrix (Dayhoff *et al.*, 1983)]. The single letter amino acid code is utilized.

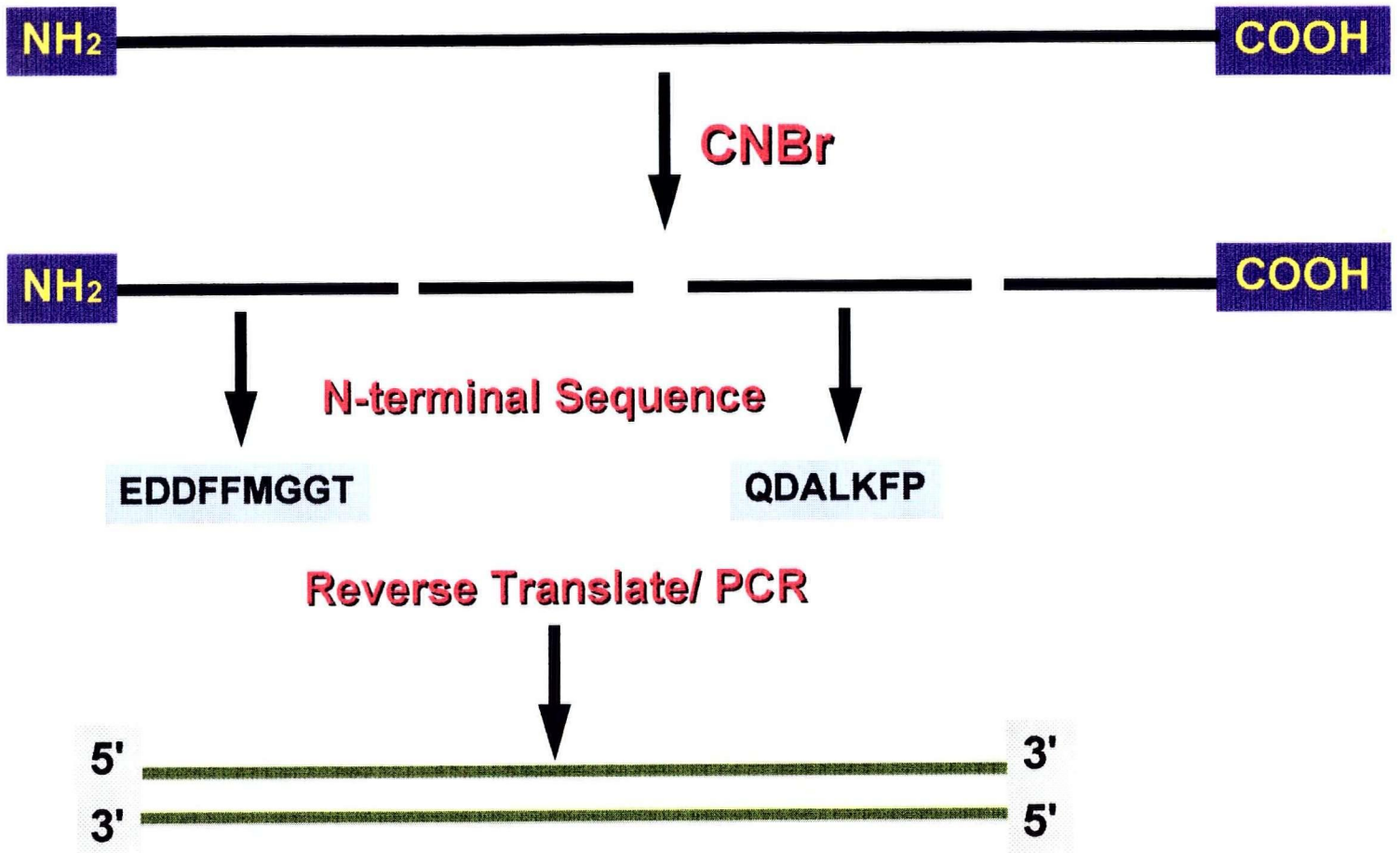

Figure 16. Schematic representation of strategy used to create probes to porin proteins.

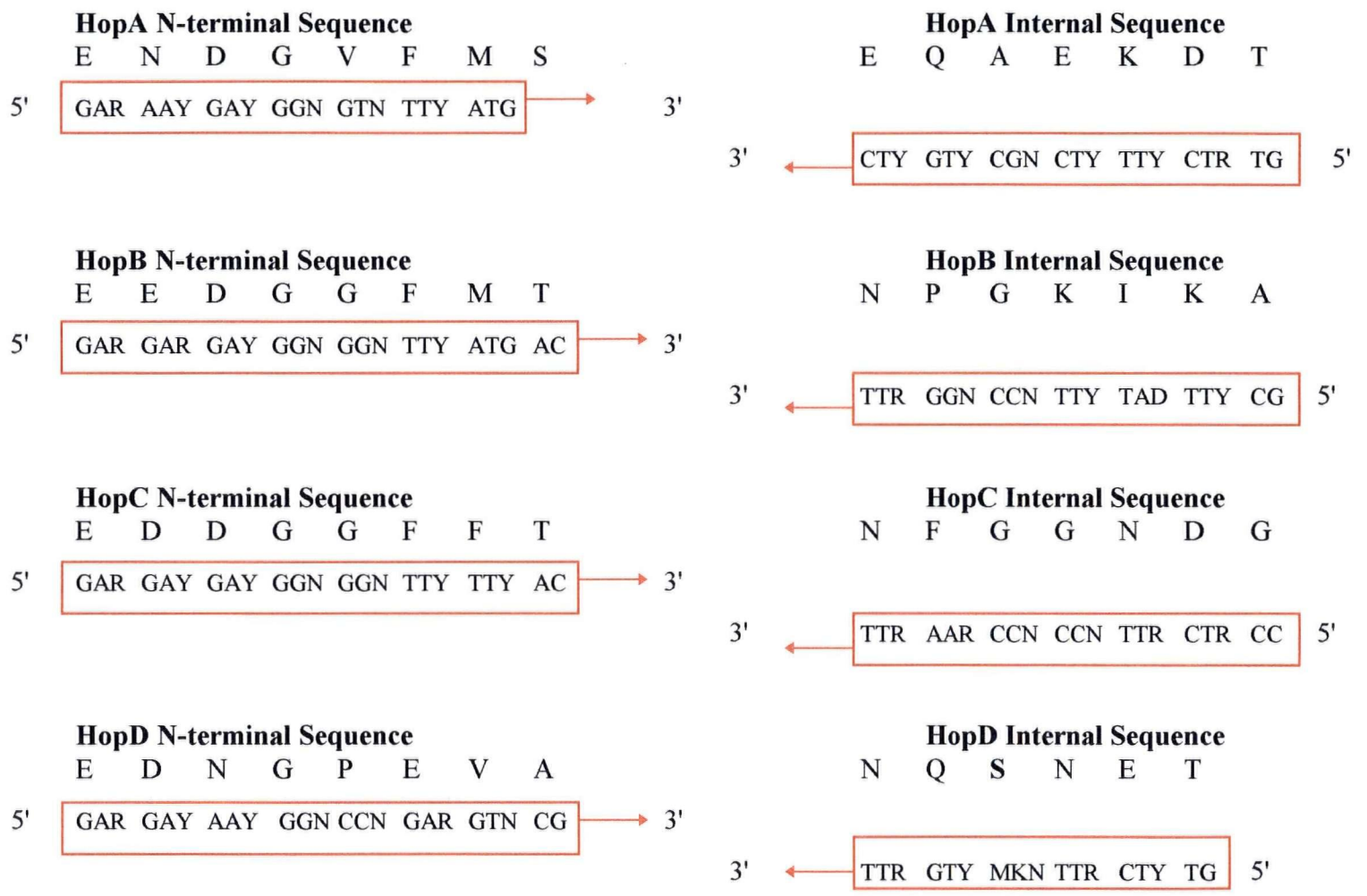

Figure 17. Reverse translated oligonucleotides used for PCR. The partial N-terminal and internal amino acid sequences are presented in the single letter code, and reverse translated nucleotide sequences are boxed in red. For nucleotides, R =A or G, Y=C or T, M =A or T, K =G or T, and N=A or C or G or T

included, as were controls using either the 5' or 3' primer alone, such that two-primer specific products could be identified apart from products produced from a single primer. It was found that PCR products were only produced when the $MgCl_2$ concentrations were above 8 mM, with the optimum concentration being 12 mM. This was a much higher concentration of $MgCl_2$ than that used for most PCR reactions which tend to require 2-4 mM $MgCl_2$. When 12 mM $MgCl_2$ was used, the concentrations of the primers, template, and dNTP's made little difference to the resultant pattern of PCR products. The PCR products obtained under the optimized conditions are shown in Figure 18. There were a number of products produced in each reaction, although only a small number of products for both *hopA* and *hopB* were two-primer specific as opposed to random products produced by a single primer. Products were obtained with HopC and HopD primers, but in neither case were there any prominent two-primer specific products which could be isolated.

Two-primer specific products obtained using *hopA* primers and *hopB* primers were isolated by gel purification using the Geneclean II kit and protocols (BIO 101, Vista, CA). The products were then reamplified and cloned into the pCRII vector (Invitrogen). The vector was then transformed into INVαF' cells, transformants containing inserts were identified, and the plasmid DNA from these clones was isolated. These clones were then sequenced, and clones encoding the N-terminal region of HopA and HopB were identified by comparing the sequences with the reverse translated protein sequences which extended

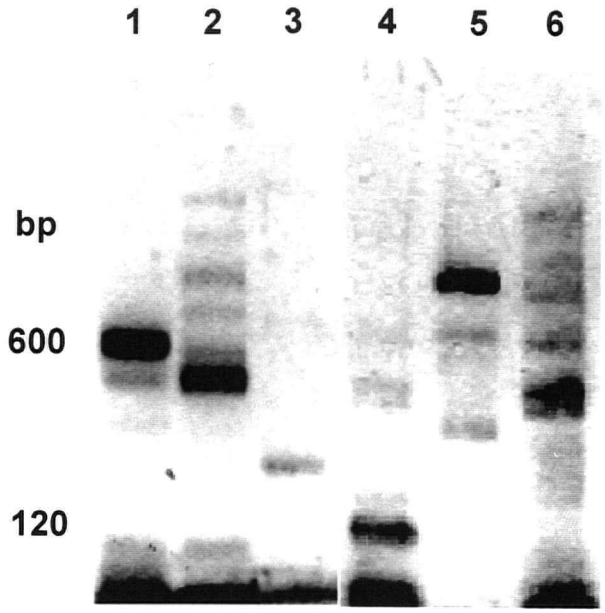

Figure 18. PCR products using *H, pylori* chromosomal DNA and primers derived from reverse translations of N-terminal and internal protein sequences. Reactions using primers corresponding to sequences from HopA are seen in the first 3 lanes, while lanes 4 to 6 represent reactions using primers for HopB. Lanes 1 and 4 are reactions using both the 5' and 3' oligonucleotide primers, 2 and 5 are reactions using only the 5' oligonucleotide primer, and 3 and 6 are reactions using only the 3' oligonucleotide primer. The size and position of standard DNA fragments are expressed as the number of base pairs and is indicated on the left.

beyond the sequence of the primers. This sequence comparison ensured that the correct products were present. In this manner, a 608 bp sequence encoding the N-terminal region of HopA and an 83 bp sequence encoding the N-terminal region of HopB were cloned. Sequence analysis using the BLAST program (Altschul *et al.*, 1990) showed no strong homologies to any other DNA sequences or to protein sequences derived from the translated nucleotide sequence. Partial sequences and sequence comparisons are shown in Figure 19. These cloned DNA sequences were randomly labeled with digoxigenin and were then used as probes to identify cosmid clones which contained the *hopA* and *hopB* genes.

D) Identification of positive cosmid clones

The *H. pylori* chromosomal DNA cosmid library was screened with probes in order to identify clones which contained the *H. pylori* porin genes. Initially, colony blots were performed on approximately 2000 colonies which had been plated out on 20 agar plates. The colonies were blotted onto positively charged nylon membranes and were then hybridized at 42°C with the degenerate oligonucleotide gene probes that had been 3' end labeled with digoxigenin. As was previously noted, after the post-hybridization washes and the detection procedure, no positive clones were identified.

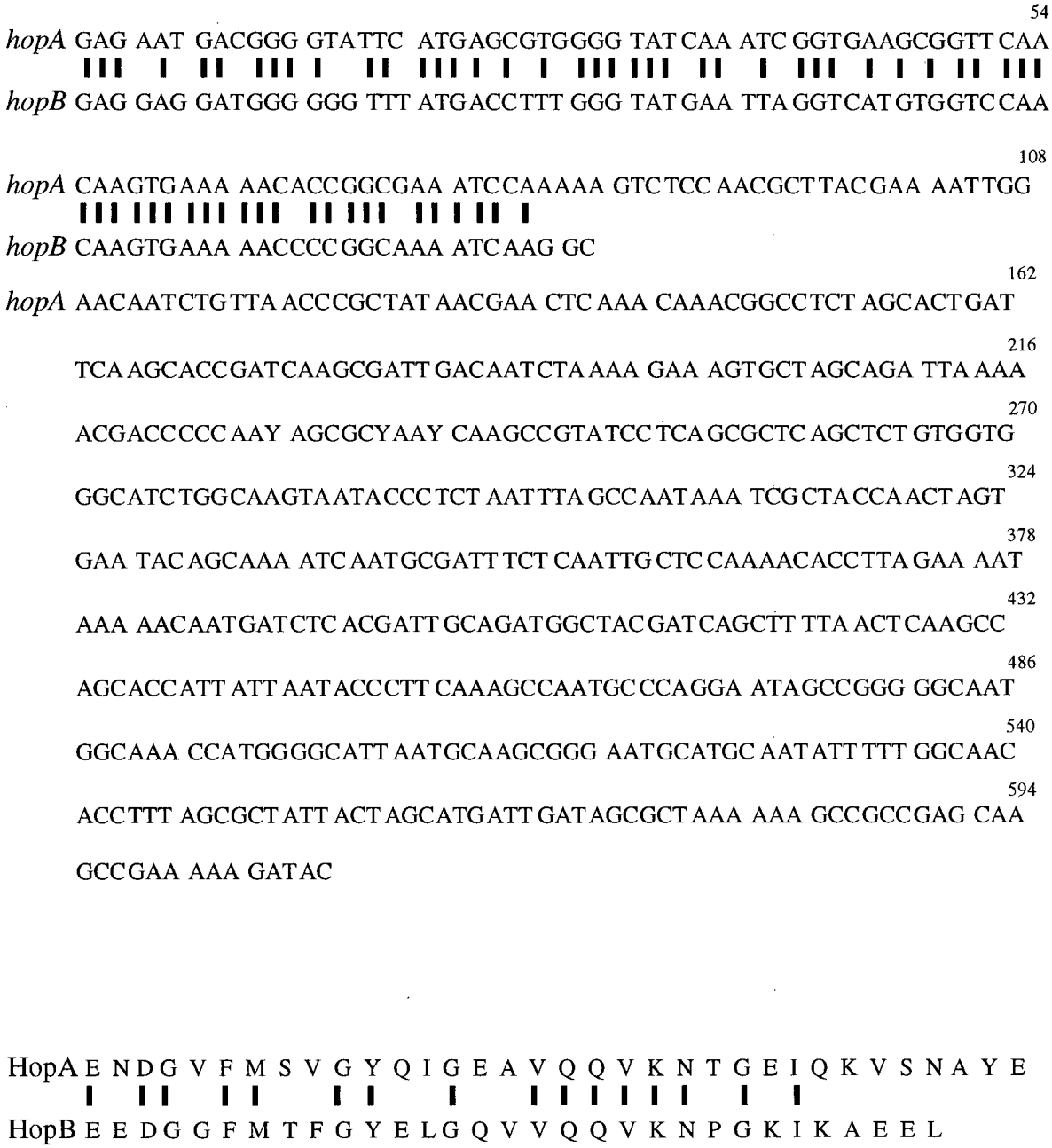

Figure 19. DNA and protein sequence comparisons of HopA and HopB. Bars represent identity between the sequences. The entire nucleotide sequences of PCR products encoding portions of the *hopA* and *hopB* genes are shown.

Efforts were later turned to using the *hopA* and *hopB* gene probes which were created by PCR and were randomly labeled with digoxigenin. 580 individual clones from the cosmid library were each inoculated into 200 µl of TYGPN in the wells of a 96 well microtitre plate. Minipreps of cosmid DNA were performed, and 5 µl of each miniprep was spotted directly onto a positively charged nylon membrane. After hybridization with the probes at 68°C, two positive clones were detected with the *hopA* probe, and two positive clones were detected with the *hopB* probe. Further analysis showed that one clone (clone 321) was identified by both probes. Thus, three clones were isolated: clone 321, which was identified with both the *hopA* and *hopB* probes, clone 322, which was identified by only the *hopA* probe, and clone 451, which was identified by only the *hopB* probe. When the positive clones were analyzed by Southern blotting (Figure 20), it was evident that the *hopB* probe also hybridized with clone 322 (Figure 20, lanes 5 to 8 of *hopB* probed blot), even though it did not originally appear to hybridize with that particular clone. In addition, the *hopB* probe hybridized with the same fragments as the *hopA* probe. The *hopA* probe did not show any reaction with clone 451 (lanes 9 to 12 in Figure 20).

Chromosomal DNA from two different *H. pylori* strains (CCUG 17874 and 5284) was isolated and digested with BamHI or EcoRI, and hybridizations were done using the PCR generated *hopA* and *hopB* probes. The resulting blots showed hybridization patterns that were similar in both strains, which suggested that the porin genes are conserved. However, two faint bands appearing at 13 kb and 1.5 kb in EcoRI digested samples were

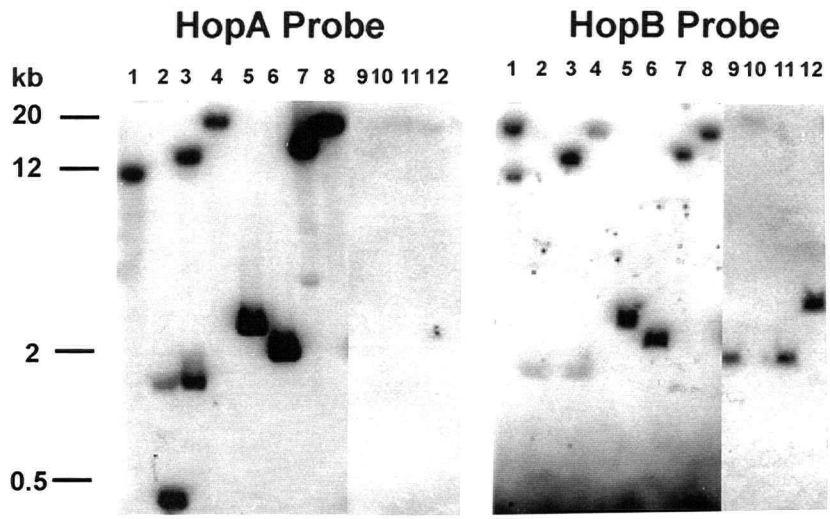

Figure 20. Southern blots of digested cosmid clones. The blots were probed with the *hopA* or *hopB* probes (as labeled) that were created using PCR. Lanes 1 to 4 are digests of clone 321, lanes 5 to 8 are digests of clone 322, and lanes 9 to 12 are digests of clone 451. Lanes 1, 5, and 9 are *Bam*HI digests, lanes 2, 6, and 10 are *Eco*RI digests, lanes 3,7, and 11 are *Hin*dIII digests, and lanes 4, 8, and 12 are double digests using *Bam*HI and *Eco*RI. The number of base pairs of the fragments (in kilobases) is indicated on the left.

present on the *hopB* probed blot of strain 5284 DNA (Figure 21, lane 4), while these bands were not detectable from strain 17874 (Figure 21 lane 2). Thus, there may be some differences between strains. The *hopB* probe hybridized with a 10 kb and a 12 kb band for EcoRI digested DNA, but the hybridization with the 12 kb band may have been a non-specific reaction with the fragment containing the *hopA* gene, as the *hopA* probed blot showed intense bands at 12 kb. The bands that appeared at 20 kb for the *hopB* probed samples may also have been a result of non-specific binding.

E) Summary

A cosmid library of *H. pylori* chromosomal DNA was created, and it was screened in order to identify clones encoding the *H. pylori* porin genes. Oligonucleotides that were 3' end labeled with DIG-ddUTP did not reveal any positive clones, so new probes were created. N-terminal amino acid sequences and internal amino acid sequences of the *H. pylori* porin proteins were obtained, and oligonucleotides were created as PCR primers after reverse translating the protein sequences. PCR reactions produced products which encoded portions of the *hopA* and *hopB* genes. These sequences were then used to screen the cosmid library. Screening with the *hopA* probe revealed two positive clones, as did screening with the *hopB* probe. One of the *hopA* clones was the same as one of the *hopB* clones, and the *hopB* probe crosshybridized with the both *hopA* clones. The *hopA* DNA probe did not hybridize with

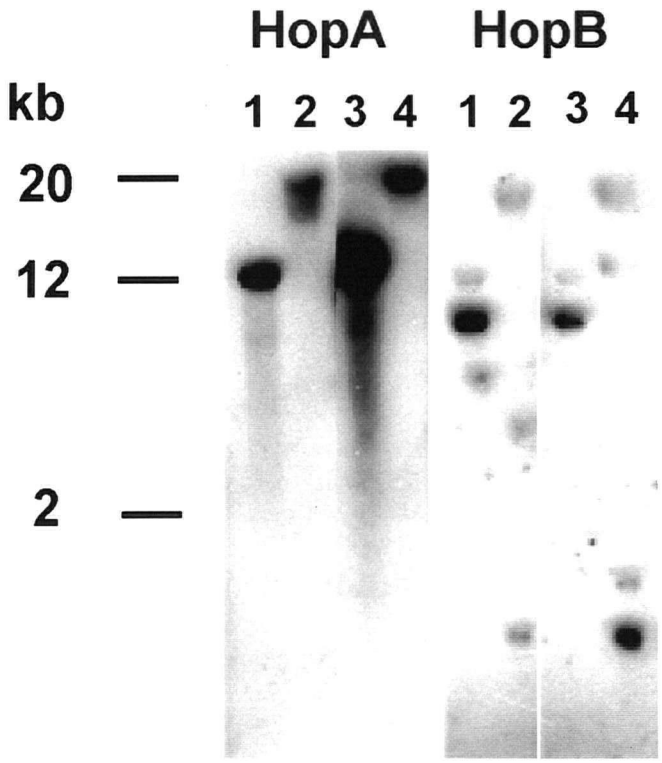

Figure 21. Southern blots of *H, pylori* chromosomal DNA. The panel labeled HopA was probed with the *hopA* probe created by PCR, and the panel labeled HopB was probed with the *hopB* probe created by PCR. In each panel lanes 1 and 2 are DNA from strain 17874, while lanes 3 and 4 are from strain 5284. Lanes 1 and 3 represent DNA digested with *Bam*HI, and lanes 2 and 4 are digests using *Eco*RI. Base pairs (kilobases) are indicated on the left.

hopB clone that was not similar to the *hopA* clones. DNA sequence analysis of the amplified regions of the *hopA* and *hopB* genes showed sequence homology along the entire sequence, although only 83 bp of the HopB sequenced was obtained.

DISCUSSION

A) Large scale growth and novel techniques and methods for manipulation of *H. pylori*

To isolate sufficient quantities of proteins which would allow for porin protein identification and purification, large masses of *H. pylori* cells were required. When these studies were initiated, a number of different media types were in use, and all of them generally utilized a rich medium that was supplemented with either blood or serum. Cells were typically grown under microaerophilic conditions which could be provided by sealed jars containing gas-generating packets. Growth in both liquid and solid media had been described, but large scale growth had not been documented. Since the production of large volumes of blood-supplemented media along with growing cells in sealed jars was rather inconvenient, experiments were undertaken to try to establish a more suitable system for growing larger batches of *H. pylori* cells.

Media supplemented with powdered hemoglobin proved to be effective for culturing *H. pylori*, and cells remained cultureable on this medium longer than they did on blood or serum supplemented media. In addition, *H. pylori* grew better on this medium in a 10% CO_2 atmosphere than they did in microaerobic conditions, even though the level of oxygen in the CO_2 incubator would be near atmospheric levels, which would be well above what would be

considered microaerobic. However, since it has been shown that *H. pylori* can be conditioned to grow aerobically (Xia *et al.,* 1994, Tompkins *et al.,* 1994), growth in a CO_2 incubator did not seem extraordinary. It is possible that the use of hemoglobin allowed cells to remain cultureable for longer periods of time because the high hemoglobin content (1%) resulted in high iron content. Iron is required for growth, and the hemoglobin could provide a long lasting source of iron.

The ability to condition *H. pylori* strains to grow under certain growth conditions is evident when one considers that some *H. pylori* strains have been conditioned to grow aerobically without elevated CO_2 levels, while other strains do not normally grow under these conditions. It is worth noting that the *H. pylori* strains which were originally grown on blood-containing medium under microaerobic conditions immediately showed vigorous growth when subcultured and grown on the hemoglobin supplemented medium in a 10% CO_2 incubator; thus, growth on hemoglobin medium in 10% CO_2 was not simply due to conditioning organisms to grow under those conditions.

The hemoglobin supplemented medium could easily be produced in large volumes, and it was much cheaper than other media supplements. Hemoglobin supplementation costs approximately $1.10 per litre of medium, whereas bovine serum would cost $5.00 per litre, bovine serum albumin would cost $45.00 per litre, and cyclodextrin would cost $51.60 per litre (prices quoted from Sigma, Mississauga, ON). Thus, this medium was not only more effective for growing *H. pylori*, but it was also very inexpensive.

H. pylori cells did not grow on media supplemented with different protein or amino acid supplements including skim milk powder, tryptone, or casamino acids. Thus, growth of cells in media containing BSA or hemoglobin was not enhanced simply due to a high protein concentration. It originally appeared that some type of blood product was specifically required for growth, as cells grew on media supplemented with blood, serum, hemoglobin, or bovine serum albumin. However, it has been shown that *H. pylori* can be cultured on media devoid of blood products, including rich base media supplemented with cyclodextrins (Olivieri *et al.,* 1993). Thus, it would appear that the role played by the cyclodextrins also duplicates some component of blood. Possible roles for these media additives may be to interfere with the action of oxygen radicals or of peroxidated fatty acids (Hazell and Graham, 1990).

In this study, liquid cultures did not grow enough cells to permit me to efficiently carry out protein isolations and purifications. Cells grown in tissue culture flasks in shallow depths of liquid, showed superior growth to cells grown in greater depths of liquid (i.e. 600 ml of liquid in a 2 l flask). It is possible that the shallow liquid depths allowed for a more efficient exchange of atmospheric gases into the medium. Growth in agitated cultures produced better results than in non-agitated cultures, but only small volumes of liquid could be used. A variable atmosphere incubator was not available, so all agitated cultures had to be maintained in sealed jars (agitated cultures did not grow in a 10% CO_2 incubator). The increased growth with the agitated cultures may also be due to greater amounts of gases

being dissolved into the medium. In any case, all liquid growth attempts resulted in relatively low cell densities. Recently, studies have shown that efficient large scale growth can be achieved in liquid cultures and in fermentors (Olivieri *et al.,* 1993, Deshpande *et al.,* 1995). Nonetheless, the methods used do not always produce results for all strains of *H. pylori*, and in most cases, a very large inoculum of cells is required for any growth. In addition, a fermentor with adaptable gas concentrations is required, and these are not available to all laboratories.

B) Identification of a putative family of porin proteins

For many Gram-negative bacteria, prospective porin proteins can be identified relatively easily by examining their outer membrane protein profiles. Porins are often among the most prominent protein species present, and they generally show a typical heat modification pattern: trimeric structures denature into monomeric subunits after heating the sample, and this can be visualized using SDS-PAGE. In the case of *H. pylori*, it was not easy to identify prospective porin proteins. No distinct major bands were present in membrane preparations other than bands corresponding to the urease subunits, and there were a large number of proteins present, unlike outer membrane preparations from typical Gram-negative bacteria such as *E. coli* (Figure 22). The large number of proteins in the *H. pylori* membrane sample may have been due, in part, to the fact that the inner and outer membranes could not be easily separated from each other, thus leaving a number of inner membrane proteins in the

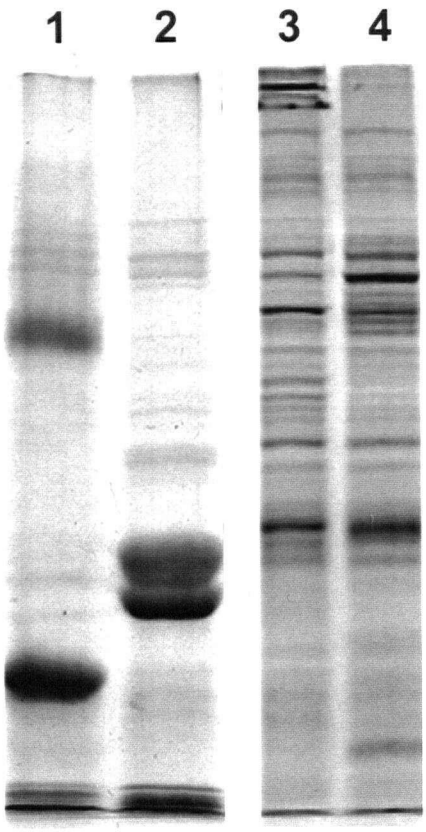

Figure 22. Comparison of *E. coli* outer membrane profile with *H. pylori* membrane profile. No major bands are seen in the *H. pylori* membrane preparations as they are in the *E. coli* outer membrane preparation. Lanes: 1) *E. coli* outer membrane preparation solubilized at 23°C, 2) *E. coli* outer membrane preparation solubilized at 95°C, 3) *H, pylori* membrane preparation solubilized at 23°C, 4) *H, pylori* membrane preparation solubilized at 95°C.

outer membrane samples, and vice versa. Sucrose gradient centrifugation, which can produce outer membranes that are relatively free of inner membrane proteins, did not give good separations with *H. pylori* membranes. In fact, it was found that the porin proteins that were eventually purified were actually found largely in what would normally be the inner membrane fraction (the less dense fraction) after centrifugation. Moreover, the less dense fraction possessed fewer different proteins, and the proteins were generally expressed at greater levels than the proteins from the more dense fraction (Figure 3). This type of profile is what would be expected for an outer membrane sample. An explanation for this phenomenon is that the density of the outer membrane must have been lower than that of gram-negative bacteria such as *E. coli*. This, in turn, may be the result of the absence of highly expressed major outer membrane proteins that are present in *E. coli*, or it could be due to the unusual LPS composition. *H. pylori* porin proteins appear to be expressed at relatively low levels, and this may be due to their specialized gastric niche. For instance, lower numbers of diffusion channels could be a mechanism by which *H. pylori* could minimize the influx of protons during its residence in the stomach. Because of the inability to efficiently separate *H. pylori* inner and outer membrane proteins, whole membrane preparations were used instead of outer membrane preparations in all purification procedures. Since the *H. pylori* porins are not highly expressed and because they were present in membrane samples amongst many other proteins, it not only made it difficult to identify the porins, but it made purification of the porins extremely difficult.

To look for prospective porins, SDS-PAGE was used in an attempt to identify trimeric complexes that denatured into monomeric subunits after heating, but none of the membrane proteins seemed to possess these characteristics. The knowledge that porins are stable β-barrel structures was then exploited to help in identifying which proteins were prospective porins. Such structures are resistant to denaturation by SDS, and only upon heating or boiling in SDS will the proteins denature and unfold from their tightly structured conformation. By examining SDS-PAGE gels of heated and unheated samples, potential β-barrel structures, which in turn would be potential porins, could be identified by looking for proteins which showed increases in apparent molecular masses after heating. Although one would normally expect the porins to be in trimeric forms that would denature into monomers after heating, porins isolated from *Pseudomonas* and *Campylobacter* species showed an alternate form of heat modification in which the monomeric β-barrels unfold (similar to the heat modifiable properties of OmpA from *E. coli.*) (Hancock and Carey, 1979, Saravolac *et al.*, 1991, Page *et al.*, 1989). Thus, it was considered that *H. pylori* may also possess porins which could be identified by observing the denaturation of monomeric units rather than the denaturation of the trimeric units.

At least 12 different heat modifiable proteins were identified, and some of these proteins showed the appearance of a multimeric complex which had broken down into subunits. However, it appeared that the multimer broke down into three subunits with different molecular masses, and the molecular masses of two of the subunits corresponded to

those of the urease subunits, while the other was approximately 18 kDa which was likely too small to be a porin. It was therefore assumed that this multimeric complex did not represent a porin. The rest of the heat modifiable proteins showed the OmpA type of heat modification, and these proteins varied in molecular mass from approximately 20 kDa to 70 kDa. All of these were potential porin candidates, but since there were three prominent proteins with molecular masses of 48 to 50 kDa, which were similar to the molecular masses of the porins isolated from *Campylobacter* species (Page *et al.*, 1989), and since *Campylobacter* species are related to *H. pylori*, these proteins were targeted as the most likely porin candidates. Therefore, purification procedures concentrated on the isolation of these three proteins.

Porin purifications often involve sequential solubilizations using a single detergent at different concentrations and with additions of salt or EDTA. However, these purifications normally use a starting material consisting of relatively pure outer membranes. These procedures did not allow for a good separation of *H. pylori* heat modifiable proteins (porin candidates) from other proteins, and this was possibly due to the fact that whole membranes were being used instead of only outer membranes. Thus, a new solubilization scheme had to be developed. After doing a number of trial solubilizations with different detergents, it was found that 1% Triton X-100 / 2 mM $MgCl_2$ and 0.5% Sarkosyl best solubilized extraneous proteins, and they solubilized only minimal amounts of the proteins of interest. This was predicted, considering that these two detergents are routinely used to solubilize inner membrane proteins. Conversely, 3% OPOE was found to be the most efficient detergent at

solubilizing the prospective porin proteins apart from other proteins. The final solubilized sample contained the HopA, B, C, D, and E proteins, with only minimal contamination from other proteins.

Following selective solubilizations of porins, liquid chromatography often enables the complete purification of the protein of interest, providing that the protein has sufficiently distinct physical and chemical properties. With *H. pylori* porins, this again was not the case. A number of porin proteins cofractionated after liquid chromatography purification attempts (HopB, C, and D), and gel purification was therefore required to separate these proteins. HopA did not appear to cofractionate with HopB, C, and D, but since it was in much lower concentration after solubilization, it simply may not have been visible in samples after anion exchange chromatography. HopE was able to be separated from HopB, C, and D, but it was not one of the proteins that was originally targeted as a porin, so it was not immediately analyzed. Given the similar molecular masses and N-terminal amino acid sequences of HopB, C, and D, it does not seem unreasonable that these proteins would have similar purification properties, and this may explain why traditional purification methods could not separate them.

The N-terminal sequences of all of the porin proteins isolated from *H. pylori* CCUG 17874 were similar, and there were a number of residues that were conserved in all instances (Figure 7). Even HopE which showed a different heat modification pattern and different pore forming characteristics from the other *H. pylori* porins, maintained these conserved residues.

It appeared that the sequence homology stretched beyond the N-terminus, as some homology was seen with internal sequences that were obtained (Figure 15). Protein sequence analysis also showed that at least part of these sequences were conserved in porins from other *H. pylori* strains. N-terminal sequences from HopB and HopC from strain CCUG 5284 were identical to those from strain 17874, although the proteins from strain 5284 showed a 2 to 3 kDa greater apparent molecular mass when observed on SDS-PAGE. It is possible that there were some protein modifications that accounted for this. Consistent with this interpretation, some amino acid residues showed aberrant retention times when subjected to HPLC during protein sequencing. There also is a possibility that the *H. pylori* porin genes have undergone recombination, which could explain in principle why there were so many similar porin species within a single strain, and why there were molecular mass differences between strains. It is known that *H. pylori* is naturally competent for transformation with *H. pylori* DNA, and that the organism has a variably arranged chromosome (Haas *et al.*, 1993, Logan and Berg, 1996). This supports the plausibility that the porin diversity is caused by genetic recombination.

The expression of a number of homologous porin proteins in a single organism is a feature that appears to be unique to *H. pylori*. Other bacterial species have homologous porins (i.e. OmpF, OmpC, and PhoE of *E. coli*), but they are not usually expressed together, except for OmpF and OmpC which are inversely regulated (Mizuno and Mizushima, 1990). *Neisseria* species express different alleles of a particular porin, but again, they are not co-

expressed (Gill *et al.*, 1994). In addition, neither *E. coli, Neisseria* species, or any other bacterium are known to possess 5 homologous porins. It was unclear why *H. pylori* possessed this apparent family of porins, or whether or not each porin had a different function. It is possible that they are selectively expressed *in vivo* but that was not addressed in this study due to the lack of a relevant animal model available to me.

All of the heat modifiable proteins that were purified were shown to have porin activity. HopA, B, C, and D, formed relatively small channels with similar single channel conductances, while HopE formed relatively large channels. HopA, B, C, and D had very similar N-terminal sequences along with similar heat modification properties, so the similarity in conductances was not surprising. HopE showed less sequence homology to the other porins, and it showed only slight modification after heating, (a change in apparent molecular mass of 3 kDa was observed, instead of the 11 or 12 kDa difference with HopA, B, C, and D). In addition, there is evidence that HopE could be associated as a trimer (Doig *et al.*, 1995). Thus, these observations are consistent with the fact that HopE formed channels that were quite different from those formed by the other *H. pylori* porins. It is quite possible that HopE has a significantly different structure than HopA, B, C, and D; nonetheless, when HopE was isolated, it was functional as a monomer. HopE may be the same protein isolated by Tufano and co-workers (1994). They reported immunobiological activities of a 31 kDa porin (the same molecular mass as HopE), although they did not show that the protein they isolated actually functioned as a porin.

The single channel conductances of HopA, B, C, and D (0.25-0.36 nS in 1 M KCl) appeared to be quite low, although since these porins were functional as monomers, the conductances would range from 0.75-1.08 nS if they were assembled into trimers like other characterized porins. This is still in the low range when compared to other general diffusion porins that have been isolated, which often are in the range of 1.3 to 2.4 nS (Hancock, 1986). On the other hand, HopE showed a conductance of 1.5 nS, and this corresponds to a conductance of 4.5 nS for a trimer, which would make it a rather large channel in comparison to even OmpF trimers from *E. coli* which have a conductance of 1.8 nS. This could indicate that HopE is the major general diffusion pore in *H. pylori*, while the other porins may be substrate-specific, as most specific porins tend to have higher molecular masses and lower single channel conductances (Hancock, 1986).

HopA, B, and C had very similar apparent molecular masses (48, 49, and 50 kDa respectively) while HopD had an apparent molecular mass of 67 kDa. Nonetheless, these porins still shared sequence homology and similar channel forming properties. However, HopD did have a lower channel conductance than HopA, B, and C, and the greater molecular mass might explain this. It is known that porins often have rather variable sized external loops which may fold into the pore (Cowan *et al.* 1992, Schirmer *et al.*, 1995). It is possible that HopD may have larger external loops than HopA, B, and C. These loops may either constrict the entrance to the pore, or they may fold into the channel to form a constriction or even a binding site, and this could explain the lower conductances observed for HopD.

HopD was also different from HopB and C in that its single channel conductance did not appear to be affected by a lower pH (HopA was not tested due to a lack of sufficient amounts of purified protein). The reason for the change in conductance at lower pH values is unknown at present, although it could be extremely significant considering that *H. pylori* is found in a gastric environment. However, it is also possible that the lower pH values simply caused a change in protein conformation or a change in the charge of acidic residues lining the channel, which would in turn lead to different channel forming characteristics.

Conductance measurements for HopB, C, D, and E were performed using different salt concentrations and with different salt solutions. The conductances measured for different concentrations of KCl were roughly proportional to the salt concentration. That is, the conductance in 1.0 M KCl was approximately 1/3 of that in 3.0 M KCl. This indicated that the channels were not being saturated by higher ion concentrations, and therefore that the ions were passing through the aqueous channel relatively unhindered by interactions with the channel lumen. This was also shown when LiCl and KCH_3COO were used as the aqueous solutions. Li^+ is a cation that carries a large hydration shell, and CH_3COO^- is a large anion, compared to K^+ and Cl^- respectively. When using the larger anions and cations, one would expect a decrease in conductance for one of the above salts, when compared to the conductance in KCl, if there was a pronounced selectivity for anions or cations respectively. A decrease in conductance when using a large cation would indicate a selectivity for cations (i.e. K^+ or Li^+ carrying the current), while a decrease in conductance while using a large anion

would indicate a selectivity for anions (i.e. Cl^- or CH_3COO^- carrying the current). A comparison of conductance values in different salt solutions can only be done when conductance is corrected for by using the σ value for a particular salt solution. The σ value represents the specific conductivity of a particular aqueous solution, and by dividing the measured conductance (G) by the σ value, one obtains a ratio. The ratios of G/σ for different salt solutions can be directly compared. The values in Table XV indicated that for HopB, C, D, and E there was a 40 to 50% decrease in the G/σ ratio for KCH_3COO as compared to KCl and LiCl, and this implied that there might be a slight selectivity for anions over cations. These differences may seem significant, but not when one compares the results to those obtained for a truly selective porin, such as OprP from *Pseudomonas aeruginosa*, which shows G/σ ratio differences of greater than 100 fold for salts with large anions (Hancock *et al.,* 1982). To more directly assess ion selectivity, zero current membrane potential experiments were done using the HopE protein. These results showed that the calculated permeability of cations through the porin channels was virtually identical to the conductance of anions, and unequivocally showed that there was no ion selectivity for this protein.

C) Evidence for new classes of porin proteins

The literature which describes porin proteins has generally depicted them as trimeric structures which are composed of three identical protein subunits. There had been few exceptions to this rule. OprF and OprB of *Pseudomonas aeruginosa*, and OmpA from

Table XV. G/σ ratios for *H. pylori* porin proteins in different salt solutions

Salt	σ (mS/cm)	HopB G/σ(nm)	HopC G/σ(nm)	HopD G/σ(nm)	HopE G/σ(nm)
0.3 M KCl	34	0.35	0.32	0.26	1.32
1.0 M KCl (pH 7.0)	112	0.32	0.29	0.21	1.34
3.0 M KCl	250	0.27	0.26	0.26	1.18
1.0 M LiCl	71	0.26	0.33	0.33	1.19
1.0 M KCH_3COO	68	0.16	0.19	0.13	0.96

σ is the specific conductivity for the particular salt solution. G/σ is the ratio of average single channel conductance (G) to specific conductivity (σ).

Escherichia coli have shown pore forming ability as monomeric units (Saint *et al.*, 1993, Benz and Hancock, 1981, Saravolac *et al.*, 1991), and other published examples include porins isolated from *Campylobacter* species [*Campylobacter jejuni, Campylobacter coli,* and *Campylobacter rectus* (formerly *Wolinella recta*) (Page *et al.*, 1989, Kennell *et al.*, 1992)]. All of these monomeric porins showed the same type of heat modifiability as did the identified *Helicobacter pylori* porins, in that after heating they appeared to denature from what was likely a tightly bound β-barrel structure. This resulted in an increase in apparent molecular mass when measured using SDS-PAGE. This form of heat modification may suggest that the *H. pylori* porins were OmpA or OprF-like, but no apparent sequence homology was observed between OmpA or OprF and the *H. pylori* porins.

Since functional monomeric porins such as those from *Helicobacter pylori* had been rarely observed in other organisms, a question arose as to whether these porins were anomalous, or whether there were similar types of porins in other bacteria. The identification of functional monomeric porins from several other organisms has shown that these may be a novel class of porins different from the stable trimeric forms such as those described for the *Enterobacteriaceae* (Benz, 1985, Hancock, 1986). It is interesting to note that four of the six functional monomeric porins identified in this study (not including the *H, pylori* porins) were found in Spirochetes. In addition, another of the porins was isolated from *Aeromonas salmonicida*, which belongs to the family *Vibrionaceae*, which are generally curved shaped organisms. *Campylobacters* and *Helicobacters* are curved to spiral organisms, suggesting

that all of these spiral shaped organisms may have evolved their porins independently from organisms such as the *Enterobacteriaceae*. This may not be entirely true, however, considering that *Xenorhabdus nematophilus* and other non-spiral organisms possess proteins that form channels as monomers. *X. nematophilus* is a member of the *Enterobacteriaceae*, and the porin, OpnP, that was isolated and characterized from this organism had a high degree of sequence similarity with OmpF from *E. coli* (Forst *et al.,* 1995). OmpF forms trimers and functions as a classical trimeric porin, and because of the sequence similarity between OmpF and OpnP, it is perhaps surprising that there was no evidence indicating that OpnP from *X. nematophilus* could form trimers. However, the crystal structure of OmpF from *E. coli* (Cowan *et al.*, 1992) shows that each monomeric unit of the trimer forms its own pore. Thus, the basic monomeric structures of these porins may be quite similar, except for differences in the composition or orientation of amino acid residues which would normally be involved in interactions with adjacent monomer subunits.

Even though the *H. pylori* porins and the porins from *Treponema pallidum, Leptospira kirschneri, Borrelia burgdorferi, Aeromonas salmonicida,* and *Xenorhabdus nematophilus* were isolated as monomers, it is still possible that they form trimers *in vivo*. There is evidence obtained by SDS-PAGE analysis of porin proteins using lower concentrations of SDS that Tromp1 from *T. pallidum,* OmpL1 from *Leptospira kirschneri,* and the 39 kDa protein from *B. burgdorferi* formed trimeric structures, even though the monomers were still functional by themselves (Blanco *et al.,* 1995, Shang *et* al., 1995,

Gherardini *et al.*, unpublished results). The same holds true for HopE from *H. pylori*, as shown by chemical crosslinking studies (Doig *et al.,* 1995). However, for the rest of the porins that I studied, there is as of yet no evidence for the formation of multimeric complexes. The possibility exists that all of these porins still do function only as trimers, and that when used in the model membrane system, the monomeric units assemble into trimeric units before forming functional channels. The spontaneous folding of denatured porin proteins as well as the formation of trimers from monomers in the presence of membranes and detergent has been demonstrated (Van Gelder *et al.,* 1994). Since the *H. pylori* porins were eluted from SDS-PAGE gels into a mild detergent and solubilized in mild, non-ionic detergents prior to lipid bilayer studies (compared with the strong ionic detergent SDS used in polyacrylamide gel electrophoresis), they may have been associating into trimers, but no direct evidence was present. Chemical crosslinking could possibly determine whether reassembly was occurring, but these experiments were not done. In any case, it was the monomers which were purified from SDS-PAGE in all instances, meaning that unlike the stable trimeric porins, these porins would at best appear to form less stable trimers that readily dissociate into monomers. Because stable trimers require harsh conditions (i.e. heating in SDS) to denature them into monomers, it is not surprising that the monomers of the stable trimeric porins have usually been found to be non-functional. Distinct from the classical stable trimeric porins, it therefore appears that there may be other types of porins, including trimers

that possess only loose associations between monomer subunits, and/or porins which may function *in vivo* as monomers.

D) Possible functions for *H. pylori* porins

It is known that many porin proteins show altered expression levels when subjected to different media conditions (Brass, 1986). Thus, an attempt to identify possible *H. pylori* porin functions was made by growing the cells on numerous different media and observing whether porin expression would be changed. Initial studies using cells grown on brain heart infusion agar supplemented with either hemoglobin or blood showed differences in protein expression only with cells grown for extended periods of time. Differences in expression were also observed between cells grown on solid media and those grown in BHI/CD liquid medium. Cells grown for 3 d or 14 d showed similar protein profiles, although cells grown for 30 d showed a different profile. Cultures grown less than 3 days were not harvested because growth was not dense prior to 3 days, so the effects of log phase or early stages of growth on expression were not monitored. In membranes from 30 d cultures, fewer protein bands were visible, and the visible protein bands were less intense than those observed with samples from 3d and 14d cultures. It does not seem likely that there is actually a genuine switch in protein expression patterns at this time. Nearly all cells at 14 d were in the coccoid form and remained coccoid to 30 d. If transition from rod to cocci or a change to a stationary growth phase corresponded with a change in protein expression, it would be expected to be visible at

14 d as well. Since equal masses of cells were used in all preparations, it is possible that the different protein profile observed in 30d cultures was due to the degradation of cells and proteins after this extended time period. Cells grown in the BHI/CD liquid medium showed increases in the expression of HopA, B, and C, and a number of other proteins. The increase in expression may have be due to the lower amounts of iron that would be present in this medium, as compared to medium supplemented with blood or hemoglobin, since iron deficiency has been shown to increase the expression of certain *H. pylori* proteins (Worst *et al.*, 1995). The overexpressed proteins observed by Worst *et al.* (1995), with molecular masses of 89, 50, 48, and 27 kDa, might have been the same as the HopA, HopC (48 and 50 kDa), 90 kDa, and 28 kDa proteins that showed increased expression after growth in BHI/CD media.

After examining growth on complex media, growth and protein expression on a defined medium based on a formulation provided by Reynolds and Penn (1994) was studied. Altering concentrations of components that had previously been shown to alter porin expression in other organisms (i.e. NaCl, Mg^{2+}, phosphate, glucose) resulted in no changes in porin expression for *H. pylori*, but changing the concentration of iron in the medium did influence porin expression. The results obtained from growth in defined media were not conclusive, however, since the membrane preparations contained numerous proteins that would make it difficult to clearly identify which proteins were the porins, and since the cell

densities and amounts of proteins obtained were too low to do efficient selective solubilizations.

Because iron can be chelated by 2,2-dipyridyl, a complex medium, BHI/CD medium, which would allow higher densities of cells to grow was used, and instead of varying the amount of iron added to a sample, the amount of chelator added was varied. Increasing the concentration of 2,2-dipyridyl in the medium was associated with increased levels of HopA, B, and C in membrane preparations. Amongst other proteins showing increased expression was a protein with an apparent molecular mass of 28 kDa (the same molecular mass as HopE unheated), but this protein did not show any apparent heat modification properties. The HopA, B, and C proteins likely corresponded to the proteins in the 48 to 50 kDa range that Worst and coworkers (1995) also observed to have increased expression under conditions of iron deprivation.

Although it is not common for porins to have their expression regulated by the availability of iron, porins have been identified as being involved with iron uptake or iron binding. For instance, OmpF and OmpC from *E. coli* have been shown to bind lactoferrin (Erdei *et al.*, 1994), the P2 porin from *Haemophilus influenzae* has been implicated in the uptake of iron from hemopexin (Wong *et al.,* 1995), and OprF from *Pseudomonas aeruginosa* appears to be involved in uptake of certain siderophore-iron complexes (Meyer, 1992). The latter two examples are especially relevant considering that one of the *H. pylori* porins (HopE) possessed sequence homology to the P2 porin, and because the *H. pylori*

porins formed pores as a monomer, as does OprF. It is known that *H. pylori* can utilize lactoferrin as an iron source (Husson *et al.,* 1993), and that *H. pylori* possesses ferritin-like proteins (Doig *et al.,* 1993), but it is unknown if or how the *H. pylori* porin proteins are involved in the binding, uptake, or sequestering of iron. It should be noted that although the *H. pylori* HopA, B, and C porins appeared to be upregulated by iron deficient conditions, the porins were still expressed in the presence of iron.

It has been estimated that enteric bacteria require approximately 1 μM of iron for growth (Payne, 1988). Because *in vivo* concentrations of free iron are typically in the order of 10^{-18} M (Griffiths, 1987), the iron concentration in the dipyridyl-containing medium, which supported growth, must have been well above *in vivo* concentrations. Growth in this medium resulted in increased porin expression, so considering that *in vivo* iron concentrations would be lower than in this medium, the *H, pylori* porins would be expected to be expressed at high levels *in vivo*. Thus, the porins may play important roles in virulence and infection.

Since *H. pylori* porins were regulated by iron, this provided a means to examine the influence of the different expression levels of porin proteins on antibiotic activity against *H. pylori*. A panel of different antibiotics were used, including those which could normally be expected to traverse the membrane through porins [ampicillin, amoxicillin, (Hancock and Bell, 1988)], those that are often effluxed out of the cell [ciprofloxacin and tetracycline (Li *et al.*, 1995)] and kanamycin, which likely enters the cells through what is termed a "self promoted uptake" mechanism (Hancock and Bell, 1988). It was interesting that increased

porin expression led to slightly increased susceptibility to the β-lactam antibiotics; it is well known that β-lactams are taken up through porins, and this study is consistent with the interpretation that these antibiotics might be taken up through HopA, B, or C. Furthermore, increased porin expression also resulted in a decrease in susceptibility to tetracycline and ciprofloxacin. This could be an indication that one or more of these porin proteins was involved in some sort of efflux system (Li *et al.*, 1995), or alternatively that an efflux system involved one of the other proteins that was observed to be overexpressed in iron deficient conditions. The change in antibiotic susceptibility with increased porin expression was not large, as MIC's for ampicillin and amoxicillin increased two fold, while MIC's decreased two fold for ciprofloxacin, and four fold for tetracycline. Porin-deficient mutants of other bacteria may show as little as a two fold greater MIC for ampicillin and a 4 fold greater MIC for tetracycline(Hancock and Bell, 1988), consistent with the magnitude of the differences shown for *H. pylori*. Since HopA, B, and C all showed increased expression under iron deficient conditions, it was not possible to determine which, if any, were specifically involved in antibiotic uptake or efflux. One cannot overlook the possibility that the different iron concentrations affected the growth rates of cells and that the expression of porins may not be directly related to antibiotic susceptibility. Further studies, possibly involving mutations or deletions of one or more of the porin genes would be required to determine if the porins were directly involved in antibiotic uptake or efflux.

E) Cloning and Genetic organization of *H. pylori* porin proteins

Attempts were made to clone and sequence the *H. pylori* porin genes to gain further knowledge regarding the overall gene structure and homology between the porins, and to determine the organization of porin genes on the *H. pylori* chromosome. The primers for the PCR reactions intended to create porin probes were designed from reverse translated N-terminal and internal protein sequences. The internal protein sequences were derived from peptide fragments that were obtained after CNBr cleavage of the *H. pylori* porin proteins. The cleavage patterns showed similarities between the different porins, and this indicated that the sequences may be similar beyond that seen in the N-terminal sequences. Furthermore, sequence derived from 10 kDa bands (10 kDa bands from HopA and HopC) showed that some residues appeared to be conserved, and this again suggests that the porins are similar beyond the N-terminus. The sequence from the 8 kDa band from HopB and the 18 kDa band from HopD also showed some sequence alignment, but the minimal alignment may simply be coincidental.

The first set of probes used in an attempt to identify porin genes were 3' end-labeled degenerate oligonucleotides, and they did not reveal any positive clones in the cosmid library of *H. pylori* chromosomal DNA. The degeneracy of these sequences meant that only a small portion of the correct sequence would be labeled, and this coupled with the relative insensitivity of 3' end labeling with DIG-ddUTP was likely the reason for the negative results. Probes made from DNA fragments created by PCR reactions with *H. pylori* chromosomal

DNA proved to be much more successful. Not only was there no sequence degeneracy, but the longer length of the DNA sequences meant that the probes could be labeled to a higher specific activity.

A PCR product encoding 608 bp of the *hopA* gene and another encoding 83 bp of the *hopB* gene were used to probe the cosmid library as well as digests of *H. pylori* chromosomal DNA. Both probes identified two cosmid clones, although they both identified an identical clone (clone 321). Thus, it appeared that one clone contained *hopA* sequences (clone 322), one contained *hopB* sequences (clone 451), and one contained sequences for both genes (clone 321). However, Southern blots with digested cosmid DNA indicated that the *hopB* probe hybridized with all isolated positive clones. Since the *hopA* and *hopB* sequences are at least partly similar, it is likely that the *hopB* probe was cross-hybridizing with the *hopA* genes. On the other hand, the *hopA* probe did not hybridize with the *hopB* specific clones (clone 451), which could be interpreted as indicating that cross hybridization was not occurring. However, the *hopB* probe encoded only 83 bp of sequence, and the entire sequence was very similar to that of *hopA*. Thus, it could be expected that the *hopB* probe would be more likely to cross hybridize unless the entire gene sequences shared the same degree of similarity. Southern blots probing *H. pylori* chromosomal DNA gave further evidence that the porins may be at different chromosomal loci, as the *hopB* probe hybridized to different chromosomal DNA bands than the ones to which the *hopA* probe hybridized.

Southern blots of the chromosomal DNA from two different *H. pylori* strains gave similar hybridization patterns when probed with the *hopA* probe, but the EcoRI digested chromosomal DNA from strain 5284 showed hybridization with 12 kb and 1.5 kb bands that were not observed in a hybridization with strain 17874 DNA. It is possible that strain 5284 possessed a gene duplication, or that it possessed another porin which was similar to HopB such that the *hopB* probe would hybridize with the gene encoding that protein. In any case, it appeared that homologous genes were present in both strains.

The presence of the porin genes in different strains, as well as the expression of porin proteins in different strains (Figure 8) is evidence that *H. pylori* porins are not strain specific. However, when monoclonal antibodies were raised to *H. pylori* surface proteins (Doig and Trust, 1994) monoclonal antibodies to HopA from strain CCUG 17874 did not cross react with proteins from other strains tested, although HopE did appear to be conserved in all strains (Doig *et al.*, 1995). Strain CCUG 5284, was not tested. It is thus possible that not all of the porins are expressed in all strains. On the other hand, it is also possible that the porin proteins are similar but not identical in all strains (evidenced by the different molecular masses of porins from the two strains examined in this study). This apparent protein variability could explain why monoclonal antibodies did not detect HopA homologs in different strains. In addition, the porin genes may be present in all strains, but they might not always be expressed, and this would not be detected by monoclonal antibodies.

F) Conclusions

The work done in this study has shown that *Helicobacter pylori* possesses at least 5 proteins that function as porins. These porin proteins all have similar N-terminal protein sequences, and it suggests that they form a family of related porins. Four of the proteins, HopA, B, C, and D had similar channel forming characteristics, forming relatively small channels, and were isolated as monomers. HopE, on the other hand, formed relatively large channels. It was also isolated as a monomer, but there is evidence that it may form trimers. A number of porin proteins were isolated and characterized from other bacteria. They also showed channel activity as monomers, providing evidence for the existence of a class of monomeric porin proteins. Certain *H. pylori* porin proteins showed increased expression under conditions of iron deprivation, and they may therefore function in iron uptake. Cosmid clones encoding at least two of the porin genes were identified, and further manipulations of the porin genes may enable a better characterization of these porins' functions in iron uptake. To date, nucleotide and protein sequences that have been obtained have shown no homology to porins from other organisms, but further sequence data should allow for a better characterization of the gene structure and protein structure of these porins. It is hoped that further studies into the porin sequences, the positions of the genes on the chromosome, and their regulation may enable a complete elucidation of the reasons why this family of porins exists in *H. pylori* and how each porin functions.

REFERENCES

Altschul, S.F., W. Gish, W. Miller, E.W. Myers, and D.J. Lipman. (1990) Basic local alignment search tool. J. Mol. Biol. 215:403-410.

Appelmelk, B.J., I. Simoons-Smit, R. Negrini, A.P. Moran, G.O. Aspinall, J.G. Forte, T. De Vries, H. Quan, T. Verboom, J.J. Maaskant, P. Ghiara, E. J. Kuipers, E. Bloemena, T.M. Tadema, R.R. Townsend, K. Tyagarajan, J.M. Crothers Jr., M.A. Monteiro, A. Savio, and J. De Graaff. (1996) Potential role of molecular mimicry between *Helicobacter pylori* lipopolysaccharide and host Lewis blood group antigens in autoimmunity. Infect. Immun. 64:2031-2040.

Aronoff, S.C. (1988) Outer membrane permeability in *Pseudomonas cepacia*: diminished porin content in a beta-lactam-resistant mutant and in resistant cystic fibrosis isolates. Antimicrob. Agents Chemother. 32:1636-1639.

Aspinall, G.O., M.A. Monteiro, H. Pang, E.J. Walsh, and A.P. Moran. (1996) Lipopolysaccharide of the *Helicobacter pylori* type strain NCTC 11637 (ATCC 43504): structure of the O antigen chain and core oligosaccharide regions. Biochem. 35:2489-2497.

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Noore, J.G. Seidman, J.A. Smith, and K. Struhl. (1987) Current protocols in molecular biology. John Wiley & Sons, Toronto.

Benz, R. (1985) Porin from bacterial and mitochondrial outer membranes. Critical Reviews in Biochemistry 19:145-190.

Benz, R., and R.E.W. Hancock. (1981) Properties of the large ion-permeable pores formed from protein F of *Pseudomonas aeruginosa* in lipid bilayer membranes. Biochim. Biophys. Acta. 646:298-308.

Benz R., K. Janko, and P. Lauger. (1979) Ionic selectivity of pores formed by the matrix protein (porin) of *Escherichia coli*. Biochim. Biophys. Acta. 551:238-247.

Benz, R., A. Schmid and R.E.W. Hancock. (1985) Ion selectivity of gram-negative bacterial porins. J. Bacteriol. 162:722-727.

Blanco, D.R., C.I. Champion, M. M. Exner, H. Erdjument-Bromage, R.E.W. Hancock, P. Tempst, J.N. Miller, and M.A. Lovett. (1995) Porin activity and sequence analysis of a 31 kilodalton *Treponema pallidum* subsp. *pallidum* rare outer membrane protein (Tromp1). J. Bacteriol. 177:3556-3562.

Brass, J.M. (1986) The cell envelope of gram-negative bacteria: new aspects of its function in transport and chemotaxis. Current Topics in Microbiology and Immunology. 129:1-92.

Braun, V. (1975) Covalent lipoprotein from the outer membrane of *Escherichia coli*. Biochim. Biophys. Acta 415:335-377.

Buck, G.E., W.K. Gourley, W.K. Lee, K. Subramanyam, J.M. Latimer, and R.A. DiNuzzo. (1986) Relationship of *Campylobacter pyloridis* to gastric and peptic ulcer. J. Infect. Dis. 153:664-669.

Champion, C.I., D.R. Blanco, M.M. Exner, H. Erdjument-Bromage, R.E.W. Hancock, P. Tempst. J.N. Miller, and M.A. Lovett. Submitted.

Cover, T.L. and M.J. Blaser. (1992) Purification and characterization of the vacuolating toxin from *Helicobacter pylori*. J. Biol. Chem. 267:10570-10575.

Cowan, S.W., T. Schirmer, G. Rummel, M. Steiert, R. Ghosh, R.A. Pauptit, J.N. Jansonius, and J.P. Rosenbusch. (1992) Crystal structures explain functional properties of two *E. coli* porins. Nature (London) 358:727-733.

Cronan Jr., J.E. (1979) Phospholipid synthesis and assembly. In: Bacterial outer membranes. John Wiley and Sons, Toronto. p. 35-66.

Davies, J.K., and P. Reeves. (1975) Genetics of resistance to colicins in *Escherichia coli* K-12: cross resistance among colicins of group A. J. Bacteriol. 123:102-117.

Dayhoff, M.O., W. C. Barker, and L.T. Hunt. (1983) Establishing homologies in proteins. Methods Enzymol. 91:524-545.

de Cock, H., R.T. Hendriks, T. de Vrije, and J. Tommassen. (1990) Assembly of an *in vitro* synthesized *Escherichia coli* outer membrane porin into its stable trimeric configuration. J.Biol. Chem. 265:4646-4651.

Deltenre, M., C. Jonas, J. Otero, A. Cozzoli, P. Denis, A. Burette, and E. de Koster. (1996) Strategies for *Helicobacter pylori* eradication in 1995: a review of international and Belgian experience. J. Physiology & Pharmacology. 47:59-69.

Desai, H.G., H.H. Gill, K. Shankaran, P.R. Mehta, and S.R. Prabhu. (1991) Dental plaque: a permanent reservoir of *Helicobacter pylori*? Scand. J. Gastroenterol 26:1205-1208.

Deshpande, M., E. Calenoff and L. Daniels. (1995) Rapid large-scale growth of *Helicobacter pylori* in flasks and fermentors. Appl. Environ. Microbiol. 61:2431-2435.

Doig, P., and T.J. Trust. (1994) Identification of surface exposed outer membrane antigens of *Helicobacter pylori*. Infect. Immun. 62:4526-4533.

Doig, P., J.W. Austin, and T.J. Trust. (1993) The *Helicobacter pylori* 19.6 kDa protein is an iron-containing protein resembling ferritin. J. Bacteriol. 175:557-560.

Doig, P., M.M. Exner, R.E.W. Hancock, and T.J. Trust. (1995) Isolation and characterization of a conserved porin protein from *Helicobacter pylori*. J. Bacteriol. 177:5447-5452.

Dunn, B.E., G.P. Campbell, G. Perez-Perez, and M.J. Blaser. (1990) Purification and characterization of urease from *Helicobacter pylori*. J. Biol. Chem. 265:9464-9469.

Dunn, B.E., G.I. Perez-Perez, and M.J. Blaser. (1989) Two-dimensional gel electrophoresis and immunoblotting of *Campylobacter pylori* proteins. Infect. Immun. 57:1825-1833.

Eaton, K.A., C.L. Brooks, D.R. Morgan, and S. Krakowa. (1991) Essential role of urease in pathogenesis of gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. Infect. Immun. 59:2470-2475.

Eaton, K.A., D.R. Morgan, and S. Krakowal. (1989) *Campylobacter pylori* virulence factors in gnotobiotic piglets. Infect. Immun. 57:1119-1125.

Evans, D.J. Jr., D.G. Evans, S.S. Kirkpatrick, and D.Y. Graham. (1991) Characterization of the *Helicobacter pylori* urease and purification of its subunts. Microb. Pathogenesis. 10:15-26.

Evans, D.G., D.J. Evans, J.J. Moulds, and D.Y. Graham. (1988) N-acetylneruaminyllactose-binding fibrillar hemagglutinin of *Campylobacter pylori*: a putative colonization factor antigen. Infect. Immun. 57:1119-1125.

Erdei, J., A. Forsgren, and A.S. Naidu. (1994) Lactoferrin binds to porins OmpF and OmpC in *Escherichia coli*. Infect. Immun. 62:1236-1240.

Exner, M.M., P. Doig, T.J. Trust, and R.E.W. Hancock, (1995) Isolation and characterization of a family of porin proteins from *Helicobacter pylori* . Infect. Immun. 63:1567-1572.

Ferrero, R.L., and A. Lee. (1991) The importance of urease in acid protection for the gastric-colonizing bacteria *Helicobacter pylori* and *Helicobacter felis* sp.nov. Microb. Ecol. Health. Dis. 4:121-134.

Forst, S J. Waukau, G. Leisman, M. Exner, and R.E.W. Hancock. (1995) Functional and regulatory analysis of the OmpF-like porin, OpnP, of the symbiotic bacterium *Xenorhabdus nematophilus*. Mol Microbiol. 18:779-789.

Fox, J.G., P. Correa, N.S. Taylor, D. Zavala, E. Fontham, F. Janney, E. Rodriguez, P. Hunter, and S. Diavolitsis. (1989) *Campylobacter pylori*-associated gastritis and immune response in a population at increased risk of gastric carcinoma. Am. J. Gastroenterol. 84:775.

Freedberg, A.S., and L.E. Barron. (1940) The presence of spirochaetes in human gastric mucosa. Am.J. Dig.Dis. 7:443.

Gasbarrini G., S. Pretolani, F. Bonvicini, M.R. Gatto, E. Tonelli, F. Megraud, K. Mayo, G. Ghironzi, G. Giulianelli, and M. Grassi. (1995) A population based study of *Helicobacter pylori* infection in a European country: the San Marino Study. Relations with gastrointestinal diseases. Gut 36:838-844.

Geis, G., H. Leying, S. Suerbaum, and W. Opferkuch. (1990) Unusual fatty acid substitution in lipids and lipopolysaccharides of *Helicobacter pylori*. J. Clin Microbiol. 28:930-932.

Gherardini, F., S.-Y. Liu, M.M. Exner, and R.E.W. Hancock. Unpublished results.

Ghiara, P, M. Marchetti, M.J. Blaser, M.K.R. Tummuru, T.L. Cover, E.D. Segal, L.S. Tompkins, and R. Rappuoli. (1995) Role of the *Helicobacter pylori* virulence factors vacuolating cytotoxin, CagA, and urease in a mouse model of disease. Infect. Immun. 63:4154-4160.

Gill, M.J., J. Jayamohan, M.P. Lessing, and C.A. Ison. (1994) Naturally occurring PIA/PIB hybrids of *Neisseria gonorrhoeae*. FEMS Microbiol. Letts. 119:161-166.

Glupczynski, Y., and A. Burette. (1990) Drug Therapy for *Helicobacter pylori* infection: problems and pitfalls. Am. J. Gastroenterol. 85:1545-1551.

Glupczynski, Y., M. Labbe, M.P. Van der Linden, J.F. Nyst, and A. Burette. (1989) Lack of antibiotic compliance in patients treated for *Campylobacter pylori*-associated gastritis. Am. J. Gastroenterol. 84:1126.

Goodwin, C.S., and B.W. Worsley (eds.). (1993). *Helicobacter pylori* biology and clinical practice. CRC Press Inc., Boca Raton Florida.

Goodwin, C.S., J.A. Armstrong, T. Chilvers, M. Peters, M.D. Collins, L. Sly, W. McConnell, and W.E.S. Harper. (1989) Transfer of *C.pyloridis* and *C. mustelae* to *Helicobacter* gen. nov. as *Helicobacter pylori* comb. nov. and *Helicobacter mustelae* comb. nov. respectively. International Journal of Systematic Bacteriology 39:397-405.

Goodwin, C.S., J.A. Armstrong, and B.J. Marshall. (1987) *Campylobacter pyloridis*, gastritis, and peptic ulceration. J. Clin. Pathol. 39:353-365.

Goodwin, C.S., P. Blake, and E. Blincow. (1986) The minimum inhibitory and bactericidal concentrations of antibiotics and anti-ulcer agents against *Campylobacter pyloridis*. J. Antimicrob. Chemother. 17:309-314.

Graham, D.Y. (1989) *Campylobacter pylori* and peptic ulcer disease. Gastroenterology. 96:615-625.

Griffiths, E. (1987) The iron-uptake systems of pathogenic bacteria. p 69-138, in J.J. Bullen and E. Griffiths (ed.), "Iron and infection: molecular, physiological and clinical aspects." John Wiley & Sons, Chichester, United Kingdom.

Haas, R., T.F. Meyer, and J.P. van Putten. (1993) Aflagellated mutants of *Helicobacter pylori* generated by genetic transformation of naturally competent strains using transposon shuttle mutagenesis. Mol. Microbiol. 8:753-760.

Haas, C.E., D.E. Nix, and J.J. Schentag. (1990) In vitro selection of resistant *Helicobacter pylori* . Antimicrob. Agents Chemother. 34:1637-1641.

Hancock, R.E.W. (1987) Role of porins in outer membrane permeability. J. Bacteriol. 169:929-933.

Hancock, R.E.W. (1986) Model membrane studies of porin function. In *Bacterial outer membrane as model systems* (Inouye, M., ed.) pp 187-225. John Wiley and Sons.

Hancock, R.E.W. and A. Bell. (1988) Antibiotic uptake into gram-negative bacteria. Eur. J. Clin. Microbiol. Infect. Dis. 7:713-720.

Hancock, R.E.W. and A.M. Carey. (1979) Outer membrane of *Pseudomonas aeruginosa*: heat-2-mercaptoethanol-modifiable proteins. J. Bacteriol. 140:902-910.

Hancock, R.E.W., and H. Nikaido. (1978) Outer membranes of gram-negative bacteria. XIX. Isolation from *Pseudomonas aeruginosa* PAO1 and use in reconstitution and definition of the permeability barrier. J. Bacteriol. 136:381-390.

Hancock, R.E.W., K. Poole, and R. Benz. (1982) Outer memrane protein P of *Pseudomonas aeruginosa*: regulation by phosphate deficiency and formation of small anion-specific channels in lipid bilayer membranes. J. Bacteriol. 150:730-738.

Hancock, R.E.W., D.N. Karunaratne, and C. Bernegger-Egli. (1994) Molecular organization and structural role of outer membrane macromolecules. In "Bacterial Cell Wall" J.M. Ghuysen and R. Hakenbeck eds. Elsevier Science.

Haque, M., Y. Hirai, K. Yokota, and K. Oguma.(1995) Steryl glycosides: a characteristic feature of the *Helicobacter* spp.? J. Bacteriol. 177:5334-5337.

Hashizume, T., M. Sanada, S. Nakagawa, and N. Tanaka. (1993) Alteration in expression of *Serratia marcescens* porins associated with decreased outer membrane permeability. J. Antimicrob. Chemother. 31:21-28.

Hazell, S.L., D. Evans, and D.Y. Graham. (1991) *Helicobacter pylori* catalase. J. Gen. Micribiol. 137:57-61.

Hazell, S.L., and D.Y. Graham. (1990) Unsaturated fatty acids and viability of *Helicobacter (Campylobacter) pylori*. J. Clin. Microbiol. 28:1060-1061.

Hazell, S.L., and A. Lee. (1986) *Campylobacter pyloridis*, urease, hydrogen ion back diffusion, and gastric ulcers. Lancet 2:15-17.

Hirai, Y., M. Haque, T. Yoshida, K. Yokota, T. Yasuda, and K. Oguma. (1995) Unique cholesteryl glucosides in *Helicobacter pylori*: composition and structural analysis. J. Bacteriol. 177:5327-5333.

Hofstra H., and J. Dankert.(1979) Antigenic cross-reactivity of major outer membrane proteins in enterobacteriaceae species. J. Gen. Microbiol. 111:293-302.

Hopkins, R., P. Vial, C. Ferreccio, R. Russell, and J.G. Morris Jr. (1992) Increased *Helicobacter pylori* seropositivity among Chilean children who consume uncooked vegetables: support for a water-borne mode of transmission. Clinical Research 40:240A.

Husson, M., D. Legrand, G. Spik, and H. LeClerc. (1993) Iron acquisition by *Helicobacter pylori*: importance of human lactoferrin. Infect. Immun. 61:2694-2697.

Jeanteur, D., J.H. Lakey, and F. Pattus. (1991) The bacterial porin superfamily: sequence alignment and structure prediction. Mol. Microbiol. 5:2153-2164.

Kasahara, M. and Y. Anraku. (1974) Succinate dehydrogenase of *Escherichia coli* membrane vesicles: activation and properties of the enzyme. J. Biochem. 76:959-966.

Kelly, S.M., M.C. Pitcher, S.M. Farmery, and G.R. Gibson. (1994) Isolation of *Helicobacter pylori* from feces of patients with dyspepsia in the United Kingdom. Gastroenterology 107:1671-1674.

Kennell, W.L., C. Egli, R.E.W. Hancock, and S.C. Holt. (1992) Pore-forming ablility of major outer membrane proteins from *Wolinella recta* ATCC 33238. Infect. Immun. 60:380-384.

Kobayashi, Y., K. Okazaki, and K. Murakami. (1993) Adhesion of *Helicobacter pylori* to gastric epithelial cells in primary cultures obtained from stomachs of various animals. Infect. Immun. 61:4058-4063.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680-685.

Lambert, T., F. Megraud, G. Gerbaud, and P. Courvalin. (1986) Susceptibility of *Campylobacter pyloridis* to 20 antimicrobial agents. Antimicrob. Agents Chemother. 30:510-511.

Lazzaroni J.C., and R. Portalier. (1992) The excC gene of *Escherichia coli* K-12 required for cell envelope integrity encodes the peptidoglycan-associated lipoprotein (PAL). Mol. Microbiol. 6:735-742.

Lehninger, A. L. (1970) Biochemistry. Worth Publishers Inc.

Leunk, R.D., P.T. Johnson, B.C. David, W.G. Kraft, and D.R. Morgan. (1988) Cytotoxic activity in broth culture filtrates of *C.pylori*. J. Med. Microbiol. 26:93-99.

Lelwala-Guruge, J., F. Ascencio, A. S. Kreger, and A. Ljungh. (1993) Isolation of a sialic acid-specific surface haemagglutinin of *Helicobacter pylori* strain NCTC 11637. Int. J. Med. Microbiol. Virol. Parisitol. and Inf. Dis. 280:93-106.

Li, X.Z., H. Nikaido, and K. Poole. (1995) Role of mexA-mexB-oprM in antibiotic efflux in *Pseudomonas aeruginosa.* Antimicrob. Agents Chemother. 39:1948-1953.

Livermore, D.M. (1988) Permeation of beta-lactam antibiotics into *Escherichia coli*, *Pseudomonas aeruginosa*, and other gram-negative bacteria. Reviews of Infectious Diseases. 10:691-698.

Logan, R.P., and D.E. Berg. (1996) Genetic diversity of *Helicobacter pylori.* Lancet. 348:1462-1463.

Lugtenberg, B., and L. Van Alphen. (1983) Molecular architecture and functioning of the outer membrane of *Escherichia coli* and other gram-negative bacteria. Biochim. Biophys. Acta. 737:51-115.

Lutwyche, P., M.M. Exner, R.E.W. Hancock, and T.J. Trust. (1995) A conserved *Aeromonas salmonicida* porin provides protective immunity to rainbow trout. Infect. Immun. 63:3137-3142.

Majewski, S.I.H., and S. Goodwin. (1988) Restriction endonuclease analysis of the genome of *Campylobacter pylori* with a rapid extraction method: evidence for considerable genomic variation. J. Inf. Dis. 157:465-471.

Marshall, B.J. (1990) *Campylobacter pylori*: its link to gastritis and peptic ulcer disease. Reviews of infectious diseases. 12:S87-S93.

Marshall, B.J., J.A. Armstrong, D.B. McGechie, and R.J. Glancy. (1985) Attempt to fulfil Koch's postulates for pyloric *Campylobacter.* Med. J. Aust. 142:436-439.

Marshall B.J., L.J. Barrett, C. Prakash, R.W. McCallum, and R.L. Guerrant. (1990) Urea protects *Helicobacter (Campylobacter) pylori* from the bactericidal effect of acid. Gastroenterology. 99:697-702.

Marshall, B.J., C.S. Goodwin, J.R.Warren , R. Murray, E.D. Blincow, S.J. Blackbourn, M. Phillips, T.E. Waters, and C.R. Sanderson. (1988) Prospective double-blind trial of duodenal ulcer relapse after eradication of *Campylobacter pylori.* Lancet ii. 7-10.

Marshall, B.J., D.I. Royce, J. W. Annear, J.R. Pearman, J.R. Warren, and J.A. Armstrong. (1984) Original isolation of *C. pyloridis* from human gastric mucosa. Microbios Letts. 25:83-88.

Martin-de Argila, C., D. Boixeda, R. Canton, J.P. Gisbert, and A. Fuertes. (1995) High seroprevalence of *Helicobacter pylori* infection in coronary heart disease. Lancet. 346:310.

Matsudaira, P.T., (1993) A practical guide to protein and peptide purification for microsequencing (2nd ed.). Academic Press, San Diego.

Mattsby-Baltzer, I., Z. Mielniczuk, L. Larsson, K. Lindgren, and S. Goodwin. (1992) Lipid A in *Helicobacter pylori.* Infect. Immun. 60:4383-4387.

McNulty, C.A.M., J. Dent, and R. Wise. (1985) Susceptibility of clinical isolates of *Campylobacter pyloridis* to 11 antimicrobial agents. Antimicrob. Agents Chemother. 28:837-838.

Megraud, F. F. Bonnet, M. Garnier, and H. Lamouliatte. (1985) Characterization of *Campylobacter pyloridis* by culture, enzymatic profile, and protein content. J. Clin. Microbiol. 22:1007-1010.

Megraud, F., V. Neman-Simha, and D. Brugmann. (1992) Further evidence of the toxic effect of ammonia produced by *Helicobacter pylori* urease on human epithelial cells. Infect. Immun. 60:1858-1863.

Meyer, J.M. (1992) Exogenous siderophore-mediated iron uptake in *Pseudomonas aeruginosa*: possible involvement of porin OprF in iron translocation. J. Gen. Microbiol. 138:951-958.

Miederer, S.E., and P. Grubel. (1996). Profound increase of *Helicobacter pylori* urease activity in gastric antral mucosa at low pH. Digest. Dis. Sciences. 41:944-949.

Mizuno, T. (1979) A novel peptidoglycan-associated lipoprotein found in the cell envelope of *Pseudomonas aeruginosa* and *Escherichia coli*. J. Biochem. 86:991-1000.

Mizuno, T., and S. Mizushima. (1990) Signal transduction and gene regulation through the phosphorylation of two regulatory components: the molecular basis for the osmotic regulation of the porin genes. Mol. Microbiol. 4:1077-1082.

Mobley, H.L.T., M.J. Cortesia, L.E. Rosenthal, and B.D. Jones. (1988) Characterization of urease from *Campylobacter pylori*. J. Clin. Microbiol. 26:831-836.

Mobley, H.L.T., L.T. Hu, and P.A. Foxall. (1991) *Helicobacter pylori* urease: properties and role in pathogenesis. Scand. J. Gastroenetol. 187:39-46.

Moran, A.P. (1996) The role of lipopolysaccharide in *Helicobacter pylori* pathogenesis. Aliment. Pharmacol. Therapeutics. 10 (Suppl. 1):39-50.

Morgan, D.R., R. Freedman, C.E. Depew, and W.G. Kraft. (1987) Growth of *Campylobacter pylori* in liquid media. J. Clin. Microbiol. 25:2123-2125.

Morona, R., M. Klose, and U. Henning. (1984) *Escherichia coli* K-12 outer membrane protein (OmpA) as a bacteriophage receptor: analysis of mutant genes expressing altered proteins. J. Bacteriol. 159:570-578.

Morris, A., and G. Nicholson. (1987) Ingestion of *Campylobacter pylori* causes gastritis and raised gastric pH. Am. J. Gastroenterol. 82:192-199.

Muotiala, A.I., N. Helander, L. Pyhala, T.U. Kosunen, and A.P. Moran. (1992) Low biological activity of *Helicobacter pylori* lipopolysaccharide. Infect. Immun. 60:1714-1716.

Nakae, T., (1975) Outer membrane of *Salmonella typhimurium*: reconstitution of sucrose-permeable membrane vesicles. Bioc. Biophys. Res. Commun. 64:1224-1230.

Neuwald, A. F., J.S. Liu, and C.E. Lawrence. (1995) Gibbs motif sampling: detection of bacterial outer membrane protein repeats. Protein Science 4:1618-1632.

Newell, D.G. (1987) Identification of the outer membrane proteins of *Campylobacter pyloridis* and antigenic cross-reactivity between *C. pyloridis* and *C. jejuni*. J. Gen. Microbiol. 133:163-170.

Nikaido, H. (1988) Bacterial resistance to antibiotics as a function of outer membrane permeability. J. Antimicrob. Chemother. 22 (Suppl. A):17-22.

Nikaido, H. (1992) Porins and specific channels of bacterial outer membranes. Mol. Microbiol. 6:435-442.

Nikaido H., and E.Y. Rosenberg. (1983) Porin channels in *Escherichia coli*: studies with liposomes reconstituted from purified proteins. J. Bacteriol. 153:241-252.

Olivieri, R., M. Bugnoli, D. Armellini, S. Bianciardi, R. Rappuoli, P.F. Bayeli, L. Abate, E. Esposito, L. De Gregorio, J. Aziz, C. Basagni, and N. Figura. (1993). Growth of *Helicobacter pylori* in media containing cyclodextrins. J. Clin. Microbiol. 31:160-162.

Ormand, J.E., and N.J. Talley. (1990) *Helicobacter pylori*: controversies and an approach to management. Mayo Clin. Proc. 65:414-426.

Page, W.J., G. Huyer, M. Huyer, and E. Worobec. (1989) Characterization of the porins of *Campylobacter jejuni* and *Campylobacter coli* and implications for antibiotic susceptibility. Antimicrob. Agents Chemother. 31:121-123.

Page, W.J., and D.E. Taylor. (1988) Comparison of methods used to separate the inner and outer membranes of cell envelopes of *Campylobacter* species. J. Gen. Microbiol. 134:2925-2932.

Parsonnet, J., G.D. Friedman, D.P. Vandersteen, Y. Chang, J.H. Vogelman, N. Orentreich, and R.K. Sibley. (1991) *Helicobacter pylori* infection and risk of gastric cancer. N. Engl. J. Med. 325:1127-1131.

Patel, P., M.A. Mendall, D. Carrington, D.P. Strachan, E. Leatham, N. Nolineaux, J. Levy, C. Blakeston, C.A. Seymour, A.J. Camm, and T.C. Northfield. (1995) Association of *Helicobacter pylori* and *Chlamydia pneumonia* infections with coronary heart disease and cardiovascular risk factors. British Medical Journal 311:711-714.

Payne, S.M. (1988) Iron and virulence in the family *Enterobacteriaceae*. Crit. Rev. Microbiol. 16:81-111.

Perez-Perez, G.I., and M.J. Blaser. (1987) Conservation and diversity of *Campylobacter pyloridis* major antigens. Infect. Immun. 55:1256-1263.

Perez-Perez G.I., A.Z. Olivares, T.L. Cover, and M.J. Blaser. (1992) Characteristics of *Helicobacter pylori* variants selected for urease deficiency. Infect. Immun. 60:3658-3663.

Peterson, G.L. (1977) A simplification of the protein assay method of Lowrey *et al.* which is more generally acceptable. Anal. Biochem. 83:346-356.

Poole K., K. Krebes, C. McNally, and S. Neshat. (1993) Multiple antibiotic resistance in *Pseudomonas aeruginosa*: evidence for involvement of an efflux operon. J. Bacteriol. 175:7363-72.

Rawling, E.G., N.L. Martin, and R.E.W. Hancock. (1995) Epitope mapping of the *Pseudomonas aeruginosa* major outer membrane protein OprF. Infect. Immun. 63:38-42.

Reynolds, D.J., and C.W. Penn. (1994) Characteristics of *Helicobacter pylori* growth in a defined medium and determination if its amino acid requirements. Microbiology 140:2649-2656.

Ricci, V., C. Ciacci, R. Zarilli, P. Sommi, M.K.R. Tummuru, C.D. Blanco, C. B. Bruni, T.L. Cover, M.J. Blaser, and M. Romano. (1996) Effect of *Helicobacter pylori* on gastric epithelial cell migration and proliferation *in vitro* - role of VacA and CagA. Infect. Immun. 64:2829-2833.

Sabath, L.D., V.Lorian, D. Gerstein, P.B. Loder, and M. Finland. (1968) Enhancing effect of alkalinization of the medium on the activity of erythromycin against gram-negative bacteria. Appl. Microbiol. 16:1288-1292.

Sambrook, J., E. F. Fritsch, and T. Maniatis. (1989) Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Saravolac, E. G., N. F. Taylor, R. Benz, and R.E.W. Hancock. (1991) Purification of glucose-inducible outer membrane protein OprB of *Pseudomonas putida* and reconstitution of glucose-specific pores. J. Bacteriol. 173:4970-4976.

Saint, N., E. De, S. Julien, N. Orange, and G. Molle. (1993) Ionophore properties of OmpA of *Escherichia coli.* Biochim. Biophys. Acta. 1145:119-123.

Sarosiek, J.A., A. Slomiany, and B.L. Slomiany. (1988) Evidence of weakening of gastric mucus integrity by *C.pylori.* Scand. J. Gastroenterol. 23:585-590.

Schirmer, T., T.A. Keller, Y.F. Wang, and J.P. Rosenbusch. (1995) Structural basis for sugar translocation through maltoporin channels at 3.1 A resolution. Science 267:512-514.

Schulz, G.E. (1993) Bacterial porins: structure and function. Current Opinion in Cell Biology. 5:701-707.

Seppala, K., M. Farkkila, H. Nuutinen, K. Kakala, H. Vaananen, H. Rautelin, and T.U. Kosunen. (1992) Triple therapy of *Helicobacter pylori* infection in peptic ulcer. A 12-month follow-up study of 93 patients. Scand. J. Gastroenterol. 27:973-976.

Shahamat M., U. Mai, C. Paszko-Kolva, M. Kessel, and R.R. Colwell. (1993) Use of autoradiography to assess viability of *Helicobacter pylori* in water. Appl. Environ. Microbiol. 59:1231-1235.

Shang, E.S., M.M. Exner, T.A. Summers, C. Marinich, C.I. Champion, R.E.W. Hancock, and D.A. Haake. (1995) The rare outer membrane protein, OmpL1 of pathogenic *Leptospira* species is a heat modifiable porin. Infect. Immun. 63:3174-3181.

Singh, S.P., Y. Upshaw, T. Abdullah, S.R. Singh, and P.E. Klebba. (1992) Structural relatedness of enteric bacterial porins assessed with monoclonal antibodies to *Salmonella typhimurium* OmpD and OmpC. J. Bacteriol. 174:1965-1973.

Sipponen, P., T. U. Kosunen, J. Valle, M. Riihla, and K. Seppala. (1992) *Helicobacter pylori* infection and chronic gastritis in gastric cancer. J. Clin. Pathol. 45:319-323.

Sipponen, P., M. Siurala, and C.S. Goodwin. (1993) Histology and ultrastructure of *Helicobacter pylori* infections:gastritis, duodenitis, and peptic ulceration, and their relevance as precancerous conditions. in "*Helicobacter pylori* biology and clinical practice." C. S. Goodwin and B. W. Worsley eds. CRC Press Inc., Boca Raton, Florida pp37-62.

Sipponen, P., K. Varis, O. Fraki, U.-M Korri, K. Seppala, and M. Siurala. (1990) Cumulative 10-year risk of symptomatic duodenal and gastric ulcer in patients with or without chronic gastritis. A clinical follow-up study of 454 outpatients. Scand. J. Gastroenterol. 25:966-973.

Siurala, M., P. Sipponen, and M. Kekki. (1985) Chronic gastritis: dynamic and clinical aspects. Scand. J. Gastroenterol. 20(suppl.109):69-76.

Skurray, R.A., R.E.W. Hancock, and P. Reeves. (1974) Con$^-$-mutants: class of mutants in *Escherichia coli* K-12 lacking a major cell wall protein and defective in conjugation and adsorption of a bacteriophage. J. Bacteriol. 119:726-735.

Smoot, D.T., H.L.T. Mobley, G.R. Chippendale, J.F. Lewison, and J.H. Resau. (1990) *Helicobacter pylori* urease activity is toxic to human gastric epithelial cells. Infect. Immun. 58:1992-1994

Staskawicz B., D. Dahlbeck, N. Keen, and C. Napoli. (1987) Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. J. Bacteriol. 169:5789-5794.

Sugawara, E. and H. Nikaido. (1992) Pore-forming activity of OmpA protein of *Escherichia coli*. J. Biol. Chem. 267:2507-2511.

Tamassy K., L. Simon, and M. Francis. (1995) Epidemiology of *Helicobacter pylori* infection in Hungary (comparative sero-epidemiologic study). Orvosi Hetilap. 136:1387-1391.

Tokunaga, M., H. Okunaga, and T.Nakae. (1979) The outer membrane permeability of Gram-negative bacteria: determination of permeability rate in reconstituted vesicle membranes. FEBS Lett. 106:85-88.

Tompkins, D.S., J. Dave, and N.P. Mapstone. (1994) Adaptation of *Helicobacter pylori* to aerobic growth. Eur. J. Clin.Microbiol. Infect. Dis. 13:409-412.

Trias, J., and H.Nikaido. (1990) Outer membrane protein D2 catalyzes facilitated diffusion of carbapenem and penem through the outer membrane of *Pseudomonas aeruginosa*; Antimicrob. Agents Chemother. 34:52-57.

Tufano, M.A., F. Rossano, P. Catalanotti, G. Liguori, C. Capasso, M.T. Ceccarelli, and P. Marinelli. (1994) Immunobiological activities of *Helicobacter pylori* porins. Infect. Immun. 62:1392-1399.

Tummuru, M.K.R., T.L. Cover, and M.J. Blaser. (1993) Cloning and expression of a high-molecular-mass major antigen of *Helicobacter pylori*: evidence of linkage to cytotoxin production. Infect. Immun. 61:1799-1809.

Van Gelder, P., H. de Cock, and J. Tommassen. (1994) Detergent-induced folding of the outer-membrane protein PhoE, a pore protein induced by phosphate limitation. Eur. J. Biochem. 226:783-787.

Veldhuyzen van Zanten, S.J.O., J. Goldie, J. Hollingsworth, C. Silletti, H. Richardson, and R.H. Hunt. (1992) Secretion of intravenously administered antibiotics in gastric juice: implications for management of *Helicobacter pylori*. J. Clin. Pathol. 45:225-227.

Vogel., H., and F. Jahnig. (1986) Models for the structure of outer-membrane proteins of *Escherichia coli* derived from raman spectroscopy and prediction methods. J. Mol. Biol. 190:191-199.

Weel, J.F.L., R.W.M. van der Hulst, Y. Gerrits, P. Roorda, M. Feller, J. Dankert, G.N.J. Tygat, and A. van der Ende. (1996) The interrelationship between cytotoxin-associated gene A, vacuolating cytotoxin, and *Helicobacter pylori*-related diseases. J. Infect. Dis. 173:1171-1175.

Weiss, M.S., and Schulz, G.E. (1992). Structure of porin refined at 1.8 A resolution. J. Mol. Biol. 227:493-509.

Weissbach, A., and J. Hurwitz. (1958) The formation of 2-keto-3-deoxyheptonic acid in extracts of *Escherichia coli* B. J. Biol. Chem. 234:705-709.

Wong, J.C., R. Patel, D. Kendall, P.W. Whitby, A. Smith, J. Holland, and P. Williams. Affinity, conservation, and surface exposure of hemopexin-binding proteins in *Haemophilus influenzae*. Infect. Immun. 63:2327-2333.

Woodruff, W.A., and R.E.W. Hancock. (1989) *Pseudomonas aeruginosa* outer membrane protein F: structural role and relationship to the *Escherichia coli* OmpA protein. J. Bacteriol. 171:3304-3309.

Worst, D.J., B.R. Otto, and J. DeGraaf. (1995) Iron-repressible outer membrane proteins of *Helicobacter pylori* involved in heme uptake. Infect. Immun. 63:4161-4165.

Wray, W., T. Boulikas, V.P. Wray, and R. Hancock. (1981) Silver staining of proteins in polyacrylamide gels. Analyt. Biochem. 118:197-203.

Xia, H. X., C.T. Keane, and C.A. Morain. (1994) Culture of *Helicobacter pylori* under aerobic conditions on solid media. Eur. J. Clin.Microbiol. Infect. Dis. 13:406-409.

Xiang, Z., S. Censini, B.F. Bayeli, J.L. Telford, N. Figura, R. Rappuoli, and A. Covacci. (1995) Analysis and expression of CagA and VacA virulence factors in 43 strains of *Helicobacter*

pylori reveals that clinical isolates can be divided into two major types and that CagA is not necessary for expression of the vacuolating cytotoxin. Infect. Immun. 63:94-98.

Yanisch-Perron, C.J. Vieira, and J. Messing. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103-119.

Yoshimura F., and H. Nikaido. (1985) Diffusion of beta-lactam antibiotics through the porin channels of *Escherichia coli* K-12. Antimicrob. Agents Chemother. 27:84-92.