Motility and Chemotaxis Studies in *Helicobacter pylori*.

Thesis submitted for the degree of

Doctor of Philosophy

By

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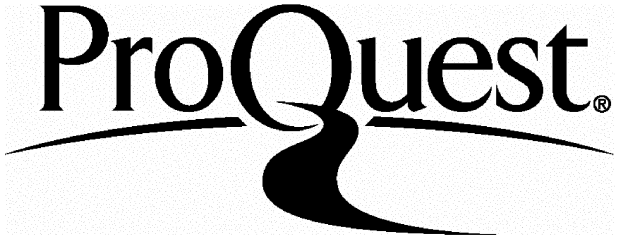

Abstract

H. pylori is one of the most common causes of chronic bacterial infection in man and its pathogenic role in the development of gastric and duodenal ulcers and gastric cancer is well documented. Chemotaxis is thought to be important in enabling *H. pylori* to reach the surface mucus layer in the stomach of infected subjects.

Little is known about the mechanism of the chemotactic response or of its role played in the motility and virulence of *H. pylori*. Therefore the first step was to amplify and clone *cheY* and *cheA* homologues from *H. pylori*. Both CheY1 and CheAY had high identity to those found in other bacterial species. *H. pylori* N6 *cheY*1, *cheAY*, *cheY*, *cheAY/Y*1 and *H. pylori* SS1 *cheY*1 and *cheAY* isogenic mutants were constructed. The pattern of the chemotactic response was studied using swarm plates, capillary tube assays and computerised motility analysis. *H. pylori* CheAY seems likely to interact with the flagellar motor to bring about motor switching, whilst the CheY1 homologue acts to terminate the response. Mucin and urea were confirmed as chemoattractants and the results of the study were combined to produced a model of chemotaxis which differs from the *E. coli* paradigm in many respects.

Finally the chemotaxis mutants were unable to colonise both the gnotobiotic piglet and mouse animal models confirming chemotaxis as a requirement for pathogenicity in *H. pylori*.

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Declaration

The conclusions reached in this thesis are my own, and are based on the experiments reported herein and published works. With the exception of those listed below, all experiments were done in the Department of Medical Microbiology, St. Bartholomew's Hospital, London.

The *H. pylori* SS1 *cheY* 1 mutant was constructed by Mr. R.A. Stabler (member of the department).

The gnotobiotic piglet animal studies were performed with Dr A. N. Rycroft of the Royal Veterinary College.

The mouse model studies were performed by Dr. A. McColm at GlaxoWellcome, Stevenage.

Abbreviations

AGS	human gastric adenocarcinoma cells
BHI	brain heart infusion
BLAST	basic local alignment search tool
CFU	colony forming unit
CW	clockwise
CCW	counter clockwise
CVS	curvilinear speed
DNA	deoxyribonucleic acid
DU	duodenal ulcer
EDTA	ethylenediamine tetra-acetic acid
FCS	fetal calf serum
HCD	highly conserved domain
HGM	hog gastric mucin
HPK	histidine protein kinase
HSP	heat shock protein
IL	interleukin
Ig	immunoglobulin
IPCRM	inverse PCR mutagenesis
IPTG	isopropyl-β-D-thiogalactopyranoside
IS	insertion sequence
kDa	kilodalton
MALT	mucosa-associated lymphoid tissue
MBP	maltose-binding protein
MCP	methyl-accepting chemotaxis protein
min	minute
MW	molecular weight
NCBI	National Centre for Biotechnology Information
NCTC	National Collection of Type Cultures
OD	optical density

OMP	outer membrane protein
ORF	open reading frame
PCR	polymerase chain reaction
PCRDOP	polymerase chain reaction with degenerate oligonucleotide primers
PFU	plaque forming unit
PHLS	Public Health Laboratory Service
pI	isoelectric point
RAPD	rapid amplification of polymorphic DNA
RFLP	restriction fragment length polymorphism
rRNA	ribosomal ribonucleic acid
RR	response regulator
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sec	second
SLV	straight line velocity
SPPCR	specific primer PCR
SS	straight swimming
Tlp	transducer-like protein
TL%	track linera percentage
t	tumbling
VPPCR	vector primer PCR
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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Chapter 1

Introduction

1.1 Genus *Helicobacter*

The stomach is a hostile environment for micro-organisms, and therefore the observations of spiral gastric microflora by Rappin in cats and dogs in 1881 and Doengis in humans in 1939 was dismissed and forgotten (Lee 1991). In 1975, Steer and Colin-Jones associated the presence of gram negative bacteria in the gastric mucosa with gastritis. However, failure to culture the organism resulted in the finding been ignored. It was not until 1983 when Warren and Marshall described the culturing of a spiral micro-organism from gastric biopsy samples showing active chronic gastritis that interest in a pathogenic role for bacteria in gastritis was rekindled. Noting their spiral morphology the organism was initially classified as a species of the genus *Campylobacter.* However in 1989, rRNA gene sequence analysis and detailed ultra structural studies, fatty acid profiles and respiratory quinones, revealed sufficient differences to place these gastric pathogens into a new genus, *Helicobacter* with *Helicobacter pylori* as the type species (Goodwin *et al* 1989). Key features ascribed to the genus *Helicobacter* were: (i) cell motility by means of a unipolar bundle of sheathed flagella; (ii) an external glycocalyx produced *in vitro* in liquid media; (iii) menaquinone-6 (MK-6) present as the major isoprenoid quinone; and (iv) G + C content of chromosomal DNA of 35-44 mol %. The *Helicobacter* genus has undergone significant expansion since 1989 and currently includes 18 named species. These have been isolated from the gastrointestinal tracts of numerous mammals, including other primates, ferrets, cats, dogs and cheetahs (see Table 1.1). Since the culturing of *H. pylori* there has been an explosive increase in interest into the microbiology of gastric bacteria. *H. pylori* is now recognised as probably one of the most common causes of chronic bacterial infection in man and its pathogenic role in the development and recurrence of gastric and duodenal ulcers and gastric cancer is well documented (Forman 1998)

1.2 Microbiology of *H. pylori*

H. pylori is a Gram negative, curved or spiral bacillus with 4-6 sheathed unipolar flagella. The bacteria are about 0.5-1.0 μm in diameter and 2.5-3.5 μm in length (Warren and Marshall 1983). *H. pylori* is a microaerophile, growing best in an atmosphere of 5% oxygen with 5-10% CO_2 on blood containing media such as Oxoid BHI agar. All cultures grow optimally at 37°C after 3-5 days (Owen 1998). *H. pylori* will grow on a suitable culture medium over a wide pH range (5.5-8.5) with good growth between pH 6.9 and 8.0, however it does not grow *in vitro* in low pH. In 2-3 day old cultures, bacteria appear slightly curved under the microscope. In 3-5 day cultures, *H. pylori* undergoes morphological change from bacillary to coccoid with an associated loss in culturability (Nilius *et al* 1993). A striking feature of *H. pylori* is potent urease activity; this and the other major biochemical properties of *H. pylori* and two other best characterised species, *H. mustalae* and *H. felis*, are shown in Table 1.2.

Species	Host	Site of isolation
H. pylori	human	gastric mucosa
H. acinonyx	cheetah	gastric mucosa
H. nemestrinae	pigtailed macaque	gastric mucosa
H. mustellae	ferret	gastric mucosa
H. sius	pigs	gastric mucosa
H. felis	cat, dog	gastric mucosa
H. bizzozeronii	dog	gastric mucosa
H. heilmannii	human	gastric mucosa
H. cinaedi	human, hamster	rectum, blood
H. fennelliae	human	rectum, blood
H. canis	dog	intestinal mucosa
H. pullorum	chickens, human	intestinal mucosa
H. pametensis	birds, pig	intestinal mucosa
H. cholecystus	hamsters	intestinal mucosa
H. hepaticus	mouse	intestinal mucosa
H. trogontum	rat	intestinal mucosa
H. bilis	mice	intestinal mucosa
H. muridarum	rat	intestinal mucosa

Table 1.1 Species of *Helicobacter* isolated from mammals. Compiled from Owen (1998).

Characersitics	*H. pylori*	*H. mustelae*	*H. felis*
Urease	+	+	+
Catalase	+	+	+
H_2S production	-	-	-
G+C content (mol%)	35-37	36	43
Ultra structure	curved to spiral	straight to curved	tightly helical
Nitrate reduction	-	+	+
Hippurate hydrolysis	-	-	-
Resistance to:			
Naladixic acid (30 μg)	+	-	+
Cephalothin (30 μg)	-	+	-
growth at 42°C	-	+	+
37°C	+	+	+
25°C	-	-	-

Table 1.2 Biochemical characteristics of *H. pylori* and related organisms. Compiled from Goodwin *et al* 1989, Paster *et al* 1991 and Owen 1998

1.3 The genome of *H. pylori*

The first reported cloning and sequencing of a *H. pylori* gene was that of urease by Clayton *et al* (1989). In less than eight years the complete genome sequence of *H. pylori* was published by Tomb *et al* (1997) (http://www.tigr.org). The strain sequenced, 26695, was originally isolated from a patient with gastritis. This strain colonises gnotobiotic piglets, elicits an immune and inflammatory response and is known to be toxigenic, plasmid free and naturally competent (Tomb *et al* 1997). It contains a single circular chromosome of 1,667,867 bp with 1,590 predicted coding sequences. 30% of the genome, ORFans, have no known homologues or any clues as to function. ORFan sequences may encode proteins unique to *H. pylori* (see Berg *et al* 1997).

H. pylori strains are highly diverse at the genetic level (see Kuipers and Blaser 1998). This has been demonstrated using arbitrarily primed PCR fingerprinting (RAPD) (Akopyanzs *et al* 1992) and RFLP analysis (Langenberg *et al* 1986, Taylor *et al* 1992). In a study by Jiang *et al* (1996) five *H. pylori* strains were compared, revealing that there is little conservation of gene location among the strains, with some genes being found in the same 25% of the genome but with little maintenance of their order. The observed diversity maybe the result of various factors. Genes, such as those involved in urease activity, show high rates of point mutations (Kansu *et al* 1996). Other genes, such as that for vacuolating cytotoxin, *vacA*, have mosaic forms (see section 1.6.3) (Atherton *et al* 1995). Additional factors that may lead to functional diversity among *H. pylori* strains are the presence of insertion sequences (Hook-Nikanne *et al* 1996) and plasmids (Kleanthous *et al* 1991).

1.4 Colonisation of the stomach and features of gastritis.

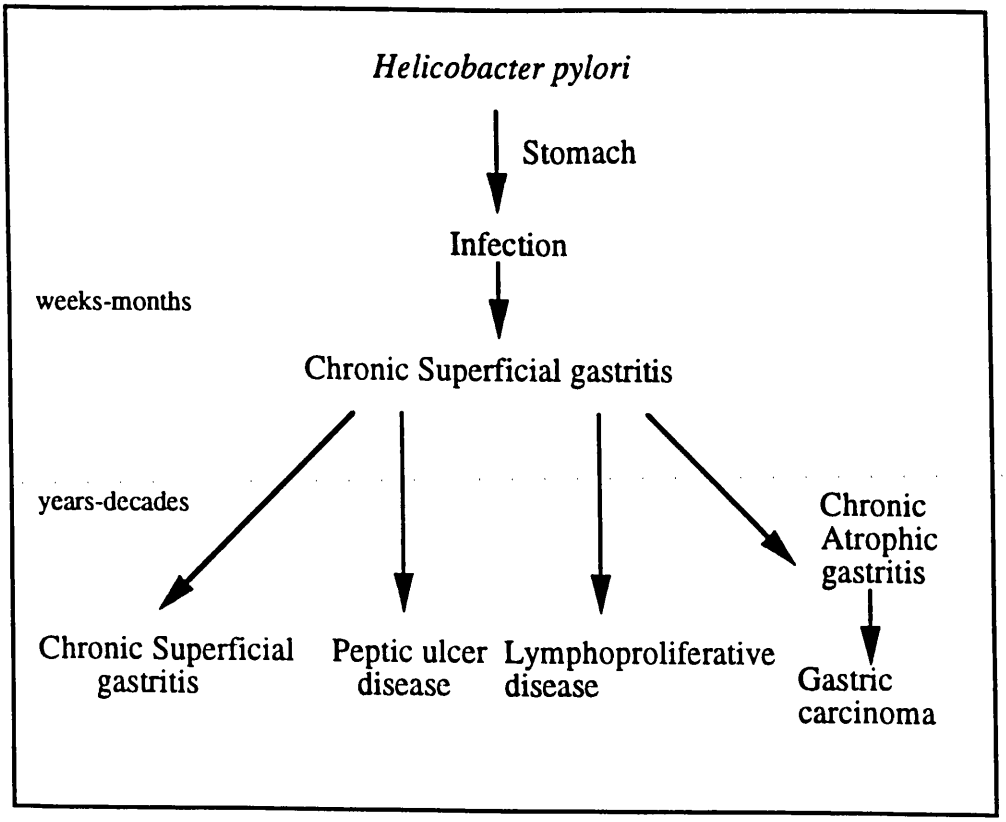
Once ingested, *H. pylori* localises to the surface of gastric epithelial cells beneath the viscous mucus layer which covers the gastric cells (Goodwin *et al* 1986). *H. pylori* is exclusively found in association with gastric type cells, but may be isolated from the duodenum, oesophagus and rectum if there is gastric metaplasia at these sites (Wyatt *et al* 1987). *H. pylori* infection induces a systemic and mucosal humoral response directed at multiple antigens (Peek and Blaser 1997). However these antibodies do not result in eradication of the bacterium from the stomach, an observation which suggests that gastric mucus may be a protected niche, relatively inaccessible to specific antibodies or their effector functions. Colonisation of the stomach by *H. pylori* is consistently accompanied by gastric inflammation comprising neutrophils, T cells, plasma cells and macrophages, along with varying degrees of epithelial cell degradation and injury (Goodwin *et al* 1986). The mucosal inflammatory response is characterised by enhanced levels

of interleukin 1L-1B, IL-2, IL-6, IL-8 and IL-10 (Peek and Blaser 1997). IL-8 has potent stimulatory properties for neutrophils, a major component found in the cellular response to *H. pylori* infection (Oppenheim *et al* 1991). This along with increased IL-8 activity in the gastric epithelium in *H. pylori* infection, suggests that IL-8 release by gastric epithelial cells may be important in initiating and regulating the inflammatory and immune responses to *H. pylori* (Crabtree *et al* 1993).

1.5 Role of *H. pylori* in human disease

The first link between *H. pylori* and human disease was the observation that this organism is almost invariably associated with chronic superficial gastritis. By now multiple lines of evidence demonstrate the causal role of *H. pylori* in this chronic inflammatory process. First, ingestion of *H. pylori* by human volunteers results in gastritis (Marshall *et al* 1985, Morris and Nicholson 1987). Second, eradication of *H. pylori* infections resolves this gastritis (Rauws *et al* 1988). There is further indirect proof supporting the causative role of the bacteria: the bacteria are found in close association with the inflamed gastric epithelium (Caselli *et al* 1989) and the observed immune response, *H. pylori* specific IgG and IgA antibodies, are seen to decrease after eradication (Oderda *et al* 1989, Seppala *et al* 1990). Chronic superficial gastritis associated with *H. pylori* infection does not produce symptoms in the majority of infected persons, but *H. pylori* infection is a significant predisposing factor for the development of peptic ulcer disease, gastric cancer and gastric lymphoma (Figure 1.1).

Figure 1.1: Clinical consequences of *H. pylori* infection.



1.5.1 Peptic ulcer disease

A peptic ulcer is a breach in the gastric or duodenal epithelium associated with acute and chronic inflammation (Calam 1998). In the absence of ulcer-inducing medications, duodenal ulcer disease occurs almost exclusively among persons infected with *H. pylori* overlying inflamed metaplastic gastric tissue (Wyatt *et al* 1990, El-Omar *et al* 1993). *H. pylori* is found in the gastric mucosa in about 60-80% of gastric ulcer patients (O' Conner *et al* 1987). In developed countries about 10% of persons colonised by *H. pylori* develop gastric or duodenal ulceration. In these patients the chance of recurrence after healing without eradication therapy is more than 30% in the first year, even with maintenance therapy using acid suppressive drugs. However, after eradication of *H. pylori*, only a few patients, and in some studies no patients, have recurrent attacks (Hopkins *et al* 1996 and Rauws and Tytgat 1990). Why colonisation with *H. pylori* leads to recurrent

peptic ulceration in some individuals but not in others, has not yet been solved. However, ulcer disease occurs more often in subjects colonised with *cagA*$^+$ *H. pylori* strains (those producing immunogenic cytotoxin associated protein) than in those with *cagA*$^-$ strains (Atherton 1998). In addition, ulcer disease appears to be more prevalent amongst patients colonised with strains having certain mosaic forms of the *vacA* gene (Atherton *et al* 1997). Bacterial heterogeneity cannot fully explain different incidence of peptic ulcer disease. Socio-economic, behavioural factors and genetic host factors almost certainly play a significant role in disease outcome (see Kuipers and Blaser 1998)

1.5.2 Gastric cancer

Chronic *H. pylori* induced inflammation can eventually lead to chronic atrophic gastritis with destruction of the gastric glands and replacement by fibrosis and metaplastic cells (Kuipers *et al* 1995). Atrophic gastritis and intestinal metaplasia, which eventually occur in half of the *H. pylori* colonised population, are associated with a 5 - 90 fold increased risk of gastric cancer, depending on the severity of the atrophy and the type of metaplasia (Sipponen *et al* 1990). Evidence that *H. pylori* is, via the sequence of atrophy and metaplasia, associated with gastric cancer originated from various studies which showed that *H. pylori* positive subjects develop gastric cancer involving the distal stomach approximately three- to sixfold more than non infected controls (Forman *et al* 1991, Nomura *et al* 1991, Parsonnet *et al* 1991). This observation was further supported by data which showed geographical associations between the prevalence of *H. pylori* and the incidence of gastric cancer in thirteen countries (The Eurogast Study Group 1993). The significance of the link between *H. pylori* and gastric cancer was demonstrated by the World Health Organisation in 1994 when *H. pylori* was formally classified a class I carcinogen (International Agency for Research on Cancer 1994). As with peptic ulcer disease, it needs to be

determined why some people develop atrophy and cancer in the presence of *H. pylori* whereas others do not. It is believed that the risk for atrophy and cancer is greater in patients with severe gastritis, as severe mucosal inflammation will sooner lead to destruction of glands and replacement by metaplasia. This hypothesis is supported by the observation that *cagA*$^+$ strains, which cause more severe inflammation (Kuipers *et al* 1996), are also associated with an approximately twofold-higher risk for atrophic gastritis and gastric cancer (Parsonnet *et al* 1997). Another factor that affects the severity and pattern of gastritis in the presence of *H. pylori* is acid production. The optimal pH for the survival and growth of *H. pylori* is pH 5 - 6, similar to that found in the antral part of the stomach, away from acid-producing parietal cells. This may explain why *H. pylori* usually causes antrally predominant gastritis. However, if acid secretion is impaired, by the use of acid supressive drugs or the presence of atrophic gastritis, *H. pylori* colonisation and gastritis in the proximal stomach increases (Logan *et al* 1995). A range of studies have supported the hypothesis that the severity and distribution of *H. pylori* gastritis increases during reduction of acid secretion (Eissele *et al* 1997, Kuipers *et al* 1995).

1.5.3 Gastric Lymphoma

A germ free stomach mucosa that is not colonised with *H. pylori* does not have lymphoid tissue. However, in the presence of *H. pylori*, lymphocytes are attracted to the gastric epithelium and lymphoid follicles can be observed. Recognition of the importance of *H. pylori* to the proliferation of gastric mucosa-associated lymphoid tissue (MALT), usually of the B-cell type, led to studies which showed that nearly all patients with such lymphoma were infected with *H. pylori* (Wotherspoon *et al* 1991). In addition the incidence of these lymphomas appeared to be related to the prevalence of *H. pylori* (Parsonnet *et al* 1994). Treatment for

H. pylori alone can lead to tumour regression in 70% of such patients after successful eradication of the organisms (Bayerdorffer *et al* 1997).

1.6 Colonisation and Pathogenic factors of *H. pylori*

H. pylori possesses determinants that allow colonisation of the gastric mucosa, evasion of the host defence and may cause damage to host tissue. Despite the availability of the complete genome sequence of *H. pylori* (Tomb *et al* 1997), many elements of pathogenesis still remain unclear at the molecular level. Urease, motility and adhesins appear to be necessary for colonisation or pathogenesis, other properties such as *cagA* and *vacA*, expressed only in a proportion of *H. pylori* strains, may explain why only some strains are associated with greater disease severity.

1.6.1 Urease

The enzyme urease is the most studied of all *H. pylori* proteins. The urease of *Helicobacter* species differs from other bacterial ureases in that it is composed of two (UreA, B)rather than three subunits (UreA, B, C). UreA and UreB have deduced molecular weights of 30 and 66 kDa respectively, which are present in a 1:1 ratio in urease (Mobley *et al* 1995). The genetic organisation of the DNA region comprising the urease structural (*ure*AB) and accessory (*ure*IEFGH) genes has been determined (Clayton *et al* 1989, Cussac *et al* 1992, Labigne *et al* 1991). The exact role of urease in the disease process is unknown, however the original hypothesis was that ammonia liberated by urease activity is vital for neutralisation of the gastric acid immediately surrounding the bacterium. It has been shown that urease negative *Helicobacter* mutants do not colonise the gastric mucosa of gnotobiotic piglets, mice or ferrets (Eaton and Krakowka 1994, Tsuda *et al* 1994 and Andrutis *et al* 1995). The vital role of urease in promoting colonisation does not appear to be simply due to its effect on the acid environment of the stomach as urease negative strains were seen to be unable to colonise achlorhydric piglets

(Eaton and Krakowka 1994). Additionally it is unlikely that urease plays a direct role in adhesion, since the adherence of the bacteria to the gastric cells is not affected by *ureB* disruption (Clyne and Drumm 1996).

Another unusual feature of the urease of *H. pylori* is that it is associated with the bacterial surface *in vivo* (Dunn *et al* 1997). Due to the absence of leader peptide sequences in the UreA and UreB polypeptides (Labigne *et al* 1991) it has been proposed that autolysis of some bacteria release urease and other cytoplasmic proteins which are subsequently absorbed onto the surface of the remaining viable bacterium (Phandis *et al* 1996). Scott *et al* (1998) also claimed that external urease is produced mostly by cell lysis, and that internal urease activity defends against gastric acidity and stimulates protein synthesis at acidic pH. This theory has been challenged by Vanet and Labigne (1998) who demonstrated that the protein profiles of supernatants from different bacterial cultures were different, indicating that not all cytoplasmic proteins are released by non-specific autolysis, but some, such as urease are selectively released.

Urease production may also be associated with the pathogenesis of *H. pylori* associated disease. The activity of urease produces ammonia which has a cytotoxic effect on gastric epithelial cells (Dekigai *et al* 1995).

1.6.2 Adhesion

By adhering to the mucus epithelial cells and the mucus layer lining the gastric epithelium, *H. pylori* can persist despite the peristalsis and gastric emptying that occurs in the stomach (Kirschner and Blaser 1995). *H. pylori* adheres to epithelial cells and produces similar attachment/effacement morphology to that seen in enteropathogenic *E. coli* (EPEC) strains (Segal *et al* 1996). It is possible that the fraction of bacteria that adhere to the mucosal surface in the body are the ones responsible for the main pathological effects. It has been demonstrated that adherence of *H. pylori* to epithelial cells is required to stimulate the production of

cytokines such as IL-8 (Reider *et al* 1997), however, the importance of attachment/effacement mechanisms in *H. pylori* pathogenesis remains unknown. Several candidate adhesins have been identified including BabA, which binds to the Lewis b (Le^b) blood group antigen on gastric epithelial cells (Ilver *et al* 1998). However, this finding has been disputed by a study demonstrating that binding to gastric cells occurs independently of Lewis antigen expression (Clyne and Drum 1997). A recent study (Guruge *et al* 1998) using transgenic mice expressing the receptor Le^b demonstrated that the development of MALT and splenic T-cell responses to *H. pylori* was increased in infected transgenic mice, implying that bacterial adherence to the epithelium promotes antigen uptake and the generation of antigenic-specific immune responses (see Dorrell *et al* 1998). Other adhesins expressed by *H. pylori* includes the neutrophil activating protein (NAP) which mediates adhesion to sulfated carbohydrates (Namavar *et al* 1998), a glycolipid-binding adhesin resembling the exoenzyme-S of *Pseudomonas aeruginosa* (Lingwood *et al* 1993) and at least one hemagglutinin (Huang *et al* 1992). A large number of lipoproteins and outer membrane proteins with unknown function have been found in the genome sequence of *H. pylori* 26695 (Tomb *et al* 1997). It is likely that some of these are also involved in adhesion.

1.6.3 Vacuolating cytotoxin

Cytotoxic *H. pylori* strains secrete a protein which induces the formation of vacuoles in the cytoplasm of mammalian cell lines *in vitro* (Leunk *et al* 1988), and causes gastric epithelial damage when given intragastrically to mice (Telford *et al* 1994). Following purification of the vacuolating cytotoxin (Cover and Blaser 1992), the gene *vacA* was cloned and sequenced simultaneously by four groups (Cover *et al* 1994, Telford *et al* 1994, Schmitt and Hass 1994 and Phandis *et al* 1994). The gene *vacA* encodes a protoxin of 139 kDa which undergoes cleavage of both a 33-amino-acid N-terminal signal sequence and a C-terminal domain to

yield the mature ~90 kDa secreted cytotoxin (see Cover 1996). Although only about 40% of *H. pylori* strains are toxigenic, all have *vacA* (Atherton *et al* 1995). However *vacA* alleles vary between strains, and in the region encoding the second half of the signal sequence, three variants, s1a, s1b and s2 have been found. In the mid region which encodes the C-terminal portion of the final processed polypeptide, two variants, m1 and m2, are found. Each *vacA* allele can consist of any combination of the mid region and signal sequence except s2/m1 (Atherton *et al* 1995), suggesting recombination between *vacA* alleles of different strains *in vivo*. Importantly, it was shown that this diversity is related to function in that s1/m1 genotypes expressed greater cytotoxic activity *in vitro* than s1/m2 strains, whereas s2/m2 genotype are non-cytotoxic (Atherton *et al* 1997). In the same study strains with *vacA* s1a alleles were found to occur most commonly in patients with ulcers (89% in one study), and strains with *vacA* m1 alleles are associated with higher levels of epithelial damage than those with m2 alleles (Atherton *et al* 1997).

1.6.4 Cytotoxin associated protein

Most cytotoxic strains also produce a 120-140 kDa immunogenic protein called CagA. The gene, *cagA*, was cloned simultaneously by three groups (Covacci *et al* 1993, Tummuru *et al* 1993, Kleanthous 1993) and varies in size between strains depending on the number of direct repeat regions in the gene (see Atherton 1998). Strains expressing cytotoxin and CagA are designated typeI, whereas typeII bacteria do not have *cagA* and do not express the vacuolating cytotoxin (Xiang *et al* 1995). The function of CagA is unclear, however, its transcription is increased at mildly acidic pH (Karita *et al* 1996). Numerous studies have shown that infection by $CagA^+$ strains, unlike $CagA^-$ strains, produce potentially damaging inflammatory responses in infected host tissue, and induce synthesis of the proinflammatory cytotoxin IL-8 in gastric biopsies and cultured cells (Crabtree *et*

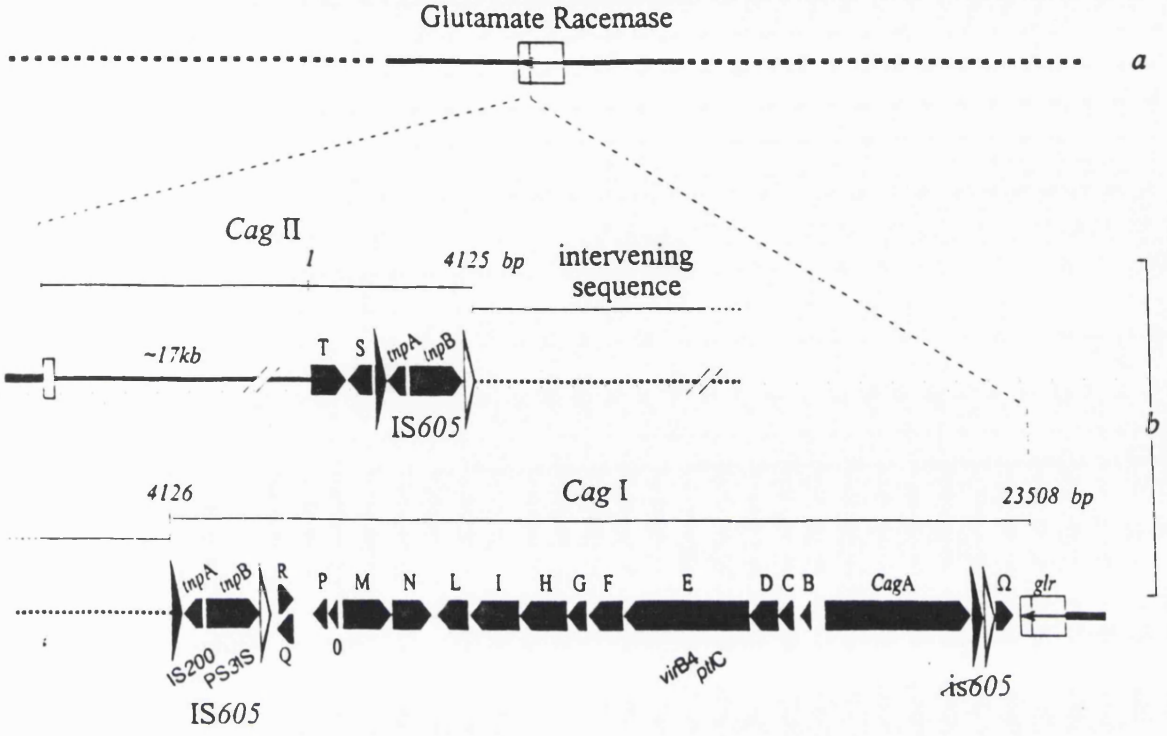


Figure 1.1a: Schemactic representation of *cag* region as deduced from analysis of CCUG 17874. The *cag* integration site with the glutamate racemase gene (*glr*) is shaded (a). *cag* structure: the putative ORFs are represented by arrows (b). Flanking the intervening sequence (dots), the IS605 have a left and right end indicated with large solid open arrowheads. The results of amino acid database searches are included. For further information see Censini *et al* 1996.

al 1994, 1995a, Sharma *et al* 1995). However, this property is unaffected by the disruption of *cagA* (Tummuru *et al* 1994, Crabtree *et al* 1995b) indicating that CagA is not directly involved in IL-8 production, but may be a marker for another protein which is responsible for epithelial cell stimulation.

Chromosome walking and mutagenesis identified two genes, *picA* and *picB* (promote induction of cytokines), upstream of *cagA*, which are absent from *cagA*$^-$ strains and are needed for IL-8 induction (Tummuru *et al* 1995). *picA* and *picB* encode polypeptides of 36 kDa and 101 kDa respectively. Of 55 clinical isolates, the *picA* and *picB* segment was conserved exclusively in CagA$^+$ strains. In addition this segment is present in all isolates from patients with duodenal ulceration compared with 59% of isolates from patients with gastric ulceration (Tummuru *et al* 1995).

Further analysis indicated that the *cagA-picB* gene cluster is part of a larger 40 kb pathogenicity island (PAI) unique to *cagA*$^+$ strains (Censini *et al* 1996). The 40 kb locus is integrated into the glutamate racemase gene and is flanked by 31 bp direct repeats. In some strains the *cag* locus is split into a right segment (*cag*I) and a left segment (*cag*II) by an insertion sequence (IS605). In other strains, *cag*I and *cag*II are separated by an intervening chromosomal sequence flanked by two IS605 sequences. This variation may be due to rearrangements which may result in strains of differing virulence. Sequencing analysis of the *cag*I region identified 19 ORFs including homologues of known proteins involved in type IV secretion systems (Censini *et al* 1996). Transposon inactivation of several of these genes was found to abolish induction of IL-8 expression in the gastric cell lines and reduce the ability of strains to induce formation of epithelial cell pedestals, phosphorylation of tyrosine and changes in the cytoskeleton (see Covacci *et al* 1997). It has therefore been proposed that the *cag*I region may encode a secretion system for export of virulence determinants (Censini *et al* 1996).

Sequencing of the *cag*II region of the PAI identified proteins with homology to numerous known virulence determinants including Ptl of *Bordetella pertussis*, VirB of *Agrobacterium tumefaciens*, Tra of conjugative plasmids, heat shock proteins, an essential GTPase (Era) and a type 1 restriction endonuclease. Some *cag* insertion mutations (VirB 10 homologue and ORF 7) blocked induction of synthesis of proinflammatory cytotoxins IL-8 in gastric epithelial cells, as did the deletion of the entire 19 kb *cag*II segment (Akopyanzs *et al* 1998).

As discussed above the link between *cagA* and vacuolating cytotoxin activity has a genetic basis: strains which are *cagA*$^+$ usually have a *vacA* s1a or b genotype, and such strains are often toxigenic (Atherton *et al* 1995, 1997) whereas strains which are *cagA*$^-$ usually have the *vacA* s2 genotype and such strains are non-toxigenic. Although it is clear that the relationship between *cagA* and certain *vacA* genotypes is genetic, it is unclear what role this link plays in the disease process. One hypothesis is that there is functional linkage between the *cag* island and the *vacA* s1 genotype such that one of these features would be advantageous only in the presence of the other: for example, a protein encoded in the *cag* region could be important in facilitating toxin export (Atherton 1998). Alternatively IL-8 production stimulated by *cagA* may increase gastric acid production or act synergistically with the cytotoxin to induce tissue damage (see McGowan *et al* 1996).

A number of studies have shown that duodenal ulceration, gastric atrophy, intestinal metaplasia, gastric cancer and MALT are more common in patients infected with *cagA*$^+$ and *vacA* (S1a/m1) genotypes (Warburton *et al* 1998, Eck *et al* 1997 and Rudi *et al* 1998). However, much remains to be elucidated about the role of the *cag* PAI and the vacuolating cytotoxin in *H. pylori* colonisation, persistence and disease.

1.6.5 Lipopolysaccharide and phase variation

Although the immunogenicity and biological activity of *H. pylori* LPS is low (Birkholz *et al* 1993), which may account for the inability of the bacteria to provoke an effective immune response, there are many studies which demonstrate that LPS contributes to the pathogenicity of *H. pylori* (Piotrowski *et al* 1997, Sakagami *et al* 1997).

H. pylori LPS has been shown to affect the quality of mucus and the secretion of pepsinogen in ulcer patients (see Moran 1995). Further work has demonstrated that the LPS of some strains may increase acid secretion by altering the histamine secretory response of enterochromaffer-like cells (Kidd *et al* 1997). Another activity has recently been attributed to the LPS of *H. pylori*. The O-polysaccharide chains of LPS of some, but not all *H. pylori* strains, exhibit mimicry of Lewis X (Le^x) and Lewis Y (Le^y) blood group antigens found in human gastric mucosa (Aspinall *et al* 1997). This molecular mimicry between LPS and the host Lewis blood group antigen has been demonstrated to cause increased inflammation and more widespread gastritis in transgenic mice (Guruge *et al* 1998). Evidence for phase-variation was recently reported for the expression of Le^x in *H. pylori* NCTC11637 (Appelmelk *et al* 1998). In this strain, the LPS which has Le^x antigenic activity, displayed a high frequency of phase variation resulting in several LPS variants in one population. Part of this observed variation has been ascribed to altered levels of expression of two genes (*fucT* and *galT*) which possess oligonucleotide repeats, and may undergo slipped strand mispairing (Appelmelk *et al* 1998). Genes with multiple short-tandem repeats located upstream are hypermutable because of slippage within the DNA repeats which results in frequent shifting into and out of frame, leading to on-off switching of the associated gene products and thereby phase or antigenic variation (Moxon *et al* 1993).

These putative homopolymeric tracts have been identified in the promoter regions of 27 genes from the *H. pylori* genome sequence data (Tomb *et al* 1997, Saunders *et al* 1998). These genes include putative LPS biosynthesis genes and cell-surface-associated genes (Saunders *et al* 1998). Therefore the phase variation proposed for Lewis antigens maybe a feature found in many virulence related *H. pylori* proteins. The results of phase-variation is a more versatile, more heterogeneous micro-organism that can cope better with a variety of environments.

1.6.6 Other putative pathogenic factors

Other putative pathogenic factors have been identified in *H. pylori*, including heat shock proteins, HspA and HspB (Macchia *et al* 1993, Suerbaum *et al* 1994). The synthesis of these essential proteins is increased in response to many environmental stresses and promotes bacterial survival. The HspA protein of *H. pylori* was shown to protect against *H. pylori* infection in the *H. felis* model (See Corthesy-Theulaz and Ferrero 1996). The identification of six *H. pylori* haemolytic genes has lead to the hypothesis that haemolysins may be important in cell damage and in the inflammatory process during infection (Drazek *et al* 1995). A final putative virulence property to be considered is the ability of *H. pylori* to form coccoid forms. Originally thought to be non-viable and thus not a causative disease factor, it has now been identified that preparations of coccoid forms can infect mice (Wang *et al* 1997) and that some coccoid forms can revert back to spiral morphology in culture media (Anderson *et al* 1997).

1.6.7 Animal models

Ever since the realisation that *H. pylori* was intimately associated with the development of gastritis and peptic ulcer disease in humans, there has been a need for a simple animal model in which the modes of pathogenicity, transmission,

immunisation and chemotherapeutic intervention can be evaluated. Various animal models have been developed for the study of *H. pylori* infection. These have included mice and ferrets which can be colonised most easily by *Helicobacter* species other than *H. pylori* (Fox *et al* 1991, Lee *et al* 1990), certain domestic cats which can carry *H. pylori* (Handt *et al* 1994), gnotobiotic piglets (Akopyanzs *et al* 1995, Krakowka *et al* 1987), particular strains of mice, which can be colonised by selected *H. pylori* strains (Marchetti *et al* 1995, McColm *et al* 1995) and several primates including the macaque monkey and the baboon (Dubois *et al* 1996). For a recent review, see Lee (1998).

1.7 Motility and Chemotaxis

Studies on a range of organisms, including *V. anguillarum*, *V. cholerae* and *P. mirabilis* (see Ottemann and Miller 1997) demonstrate the many benefits of motility and chemotaxis to host-adapted prokaryotes, including increased efficiency of nutrient acquisition, avoidance of toxic substances, the ability to translocate to preferred hosts and access optimal colonisation sites within them and dispersal in the environment during the course of transmission. Flagella mediated motility is a complex phenotype that involves significant genetic and energetic commitments by bacterial cells. In *E. coli*, approximately 49 genes and 2% of the cells total energy are required to synthesise flagella and rotate them in a regulated manner (Macnab 1996).

1.7.1 Flagellar structure and regulation in *E. coli*

The structure of a bacterial flagellum (Figure 1.2) has been extensively studied in both *E. coli* and *S. typhimurium*, by electron microscopy and genetic and biochemical analysis of flagellar mutants (Macnab 1996). The flagellum is built from the inside out with the inner-most (cell proximal) structures been assembled first, beginning with the MS ring and the basal body rod (containing the flagellar

rotor and switch). The hook, which is thought to be a universal joint, linking the filament to the basal body, is then constructed, and lastly the filament itself (Macnab 1996). Most of the flagellum lies outside the cytoplasmic membrane. The subunits which make up the filament and the hook are delivered to their sites of assembly by an export pathway specific to flagella (see Blair 1995) and occurs through a channel in the centre of the growing structure (Harshey and Toguchi 1996). Due to the energy expenditure used by the bacterial cell in the construction and control of flagella, the flagellar genes are tightly regulated by transcriptional control (Harshey and Toguchi 1996). In *E. coli* the flagellar genes are clustered into three regions on the chromosome. They are organised in a hierarchical fashion as a regulon with expression of operons at a given level affecting the expression of operons at lower levels (Figure 1.3) (Macnab 1996, Blair 1995). At the top of the flagellar gene hierarchy are the regulatory genes *flhD* and *flhC*. These genes are controlled by global cellular signals such as cyclic AMP and catabolite activator protein (glucose sensing) (Silverman and Simon 1974), phosphorylated OmpR (osmotic pressure sensing) (Shin and Park 1995) and heat shock proteins (stress sensing) (Shi *et al* 1992). The products of *flhD* and *flhC* positively control the transcription of a second level of flagellar genes, many of which encode components needed in the early stages of basal body construction. Also synthesised at this level are two regulatory gene products, FliA and FlgM, which act antagonistically. FliA encodes an alternative sigma factor that is held in an inactive state by the anti-sigma factor, FlgM, until a stage in flagellar assembly when FliA activity is needed. FlgM is a substrate of the flagellum specific export pathway and is actively pumped from the cells upon completion of the basal body and flagellar hook (Hughes *et al* 1993). Expression of the level three genes, which includes the filament, the filament cap and various motility and chemotaxis genes, thus depends on successful assembly of the basal body and flagellar hook (Macnab 1996, Blair 1995).

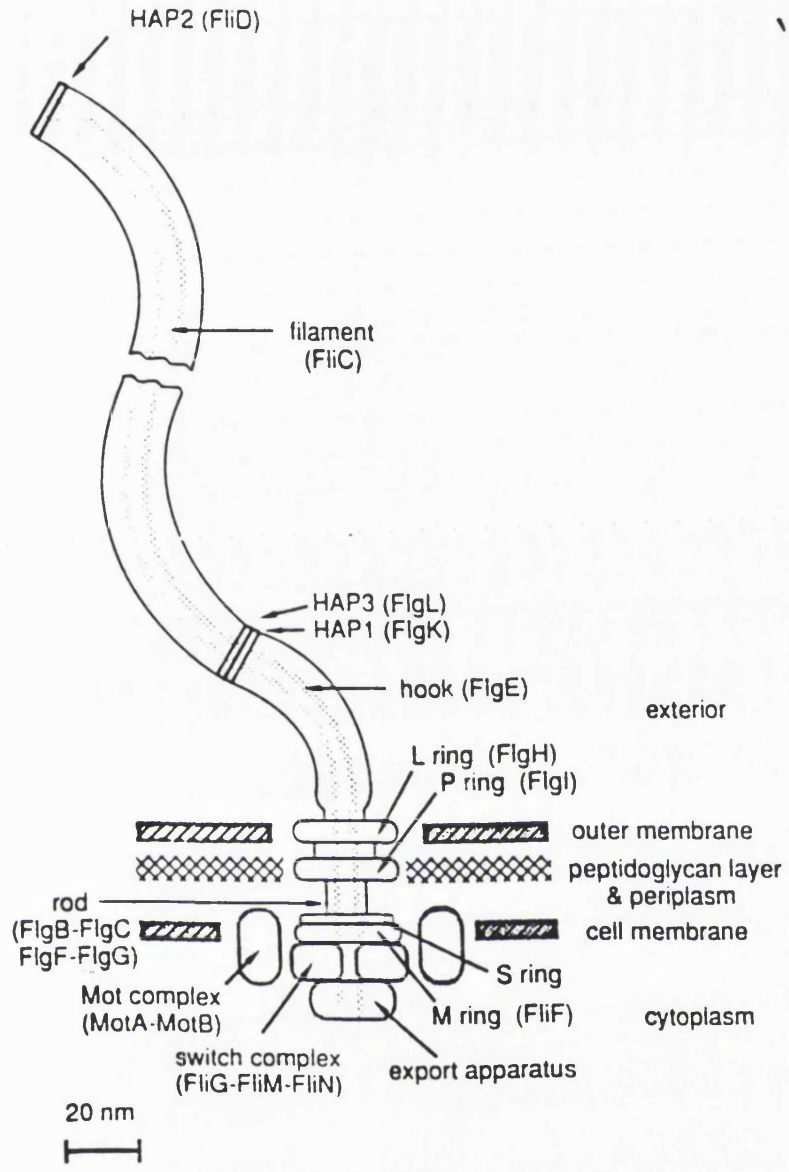


Figure 1.2: Schematic illustration of the bacterial flagellum of *E. coli*. Morphological features such as rings and rods are indicated, together with the gene products from which they are constructed. The filament is much longer than shown here. HAP, hook associated protein. Adapted from Macnab (1990).

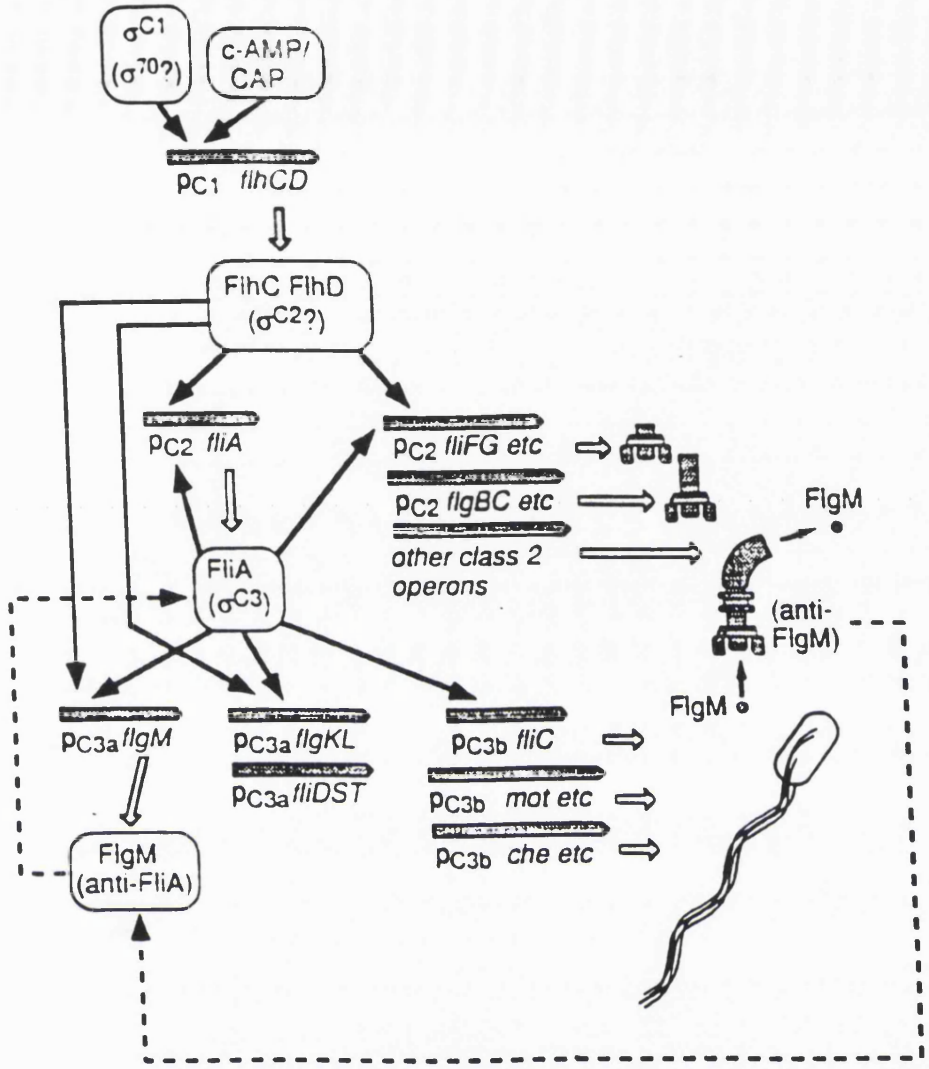


Figure 1.3: Regulation of transcription of the flagellar genes. The flagellar operons fall into a hierachy of classes of expression (see text). Operons are indicated by stippled bars, translation to products is indicated by open white arrows, positive regulatory arrows are indicated by solid arrows, and negative regulatory controls are indicated by dashed arrows. Promoters of different classes are indicated as pC1 (promoter of class 1 operon), etc., and the σ factors for transcription initiationare indicated as σ^{c1}, etc. Adapted from Macnab (1990).

1.7.2 Flagella structure and regulation in *H. pylori*.

Motility is required for colonisation of the human intestine by *H. pylori* (Eaton *et al* 1992), and is mediated by a unipolar bundle of 4-6 flagella (Goodwin *et al* 1986). At least forty proteins identified by homology in the *H. pylori* genome appear to be involved in the regulation, secretion and assembly of the flagellar architecture (Tomb *et al* 1997). To date, only *fla*A, *fla*B and *flg*E, have had functions assigned to them by mutational analysis.

The filaments of the flagella are polymers of two peptide subunits, the flagellins FlaA and FlaB. The filaments are covered by a bilayered membrane (Geis *et al* 1993) that is a continuation of the cell's outer membrane and which terminates in a bulb of unknown structure. The suggested roles of the sheath have included the protection of the filament from depolymerisation by gastric acid (Suerbaum *et al* 1993) and adhesion to the gastric mucosa (Luke and Penn 1995). The 29 kDa HpaA protein was shown by immunogold electron microscopy and mutational analysis to be a flagellar sheath protein (Luke and Penn 1995, Jones *et al* 1997). An *hpaA* mutant was able to adhere to AGS cells to the same extent as the wild type demonstrating that *hpaA* is not an adhesin (Jones *et al* 1997). The genes for both the major flagellin, FlaA (Leying *et al* 1992) and minor flagellin, FlaB (Suerbaum *et al* 1993) have been cloned and both single and double mutants have been constructed by reverse genetics (Josenhans *et al* 1995). Analysis of these mutants has demonstrated that both flagellar genes of *H. pylori* are required for full motility (Josenhans *et al* 1995) and establishment of persistent infection of the gnotobiotic piglet and mouse animal models (Eaton *et al* 1996, Danon and Eaton 1998). The *H. pylori flaA* and *flaB* genes are unlinked on the chromosome and are preceded by different promoters, σ^{28} and σ^{54} respectively (Labigne and deReuse 1996). Provisional reporter gene studies have suggested that the genes can be regulated differently by environmental conditions (Josenhans *et al* 1995b).

Another structural component of the *H. pylori* flagella, the flagellar hook (FlgE) has been purified and the gene cloned (O'Toole *et al* 1994). Mutation of *flgE* resulted in the loss of the flagellar hook and the flagellar filaments and FlaA and FlaB protein were found to accumulate intracellularly (O'Toole *et al* 1994). This suggests that, like *E. coli*, the hook protein participates in the export of the flagellar apparatus, but unlike *E. coli*, *H. pylori* does not contain a feedback system which would prevent flagellar genes of the lower hierarchical levels from being expressed when a fully functional flagella cannot exist (O'Toole *et al* 1994). A model for the involvement of two genes, *flbA* and *fliI* in the regulation of biosynthesis and export of *H. pylori* flagella has been proposed (Schmitz *et al* 1997, Jenks *et al* 1997). Both of these genes are homologous to a family of motility and virulence associated genes which are involved in flagellar assembly and type III secretion of virulence factors (VanGijsegm *et al* 1993). The lack of expression of *flaA* and *flaB* genes and growth phase reduction of expression of FlgE in *flbA* mutants combined with evidence from reporter studies have suggested that FlbA could be responsible for co-ordinating the growth phase dependent regulation of flagellin gene expression (Schmitz *et al* 1997). Mutagenesis of *fliI* also disrupts the production of FlaA and FlaB, however, based on its homology to type III secretion factors and the aflagellate nature of the mutant strains, *fliI* is thought to be primarily concerned with the export of flagella (Jenks *et al* 1997). Mutagenesis and phenotypic analysis of two other flagellar components, *fliQ* and *flhB*, demonstrated that both mutants were non-motile, aflagellate, do not express the flagellin subunits FlaA or FlaB and were unable to colonise the *H. pylori* mouse model (Foynes *et al*). These results suggest the involvement of FliQ and FlhB in the export and assembly of the flagella. How *H. pylori* regulates flagellar gene expression has yet to be elucidated. However, mutagenesis of FliQ, normally expressed in other species as a class 3 gene (Macnab 1996), affects the expression of flagellar genes in *H. pylori* (Foynes *et*

al). This suggests major differences between the classical *E. coli* system of flagellar regulation and the regulating system in *H. pylori.*

1.7.3 Chemotactic response

Chemotaxis is the purposeful movement of bacteria towards attractants and away from repellents. The chemosensory system is best characterised in *E. coli* and this is the system described in this section. However, the basic principles described apply to the majority of chemotaxis systems studied.

E. coli swims by controlling the direction of flagellar rotation. Counter-clockwise (CCW) rotation of the flagella results in swimming in a straight line known as smooth swimming, whereas clockwise (CW) rotation results in an abrupt tumbling motion as a consequence of which the cell swims in a new direction. In the absence of a chemical gradient the bacterial cell displays a random walk consisting of both runs and tumbles. When an increasing concentration of an attractant or repellent is imposed, the chemosensory pathway detects the changing concentration of the chemical with time and sends a signal to the propulsion motor, which decreases the probability of a tumble event thereby lengthening the average run up the gradient. The net effect of this temporal sensing and regulation is to change the random walk into a bias walk, in which the cell tends to migrate up an attractant gradient or down a repellent gradient as it swims (Macnab 1996, Falke *et al* 1997). In *E. coli* there are six cytoplasmic proteins (CheA, CheB, CheR, CheW, CheY and CheZ) which are regulated by methyl-accepting chemotaxis proteins (MCPs). Figure 1.4 displays a scheme of their interactions.

Detection of an attractant begins when it binds to a periplasmic binding protein (e.g galactose binding protein in the case of galactose), which then binds to an inner membrane methyl-accepting chemoreceptor protein (MCP) (eg. galactose receptor, Trg). Alternatively some attractants bind directly to the appropriate MCP. There are five monomeric soluble periplasmic proteins found in *E. coli*:

maltose-, galactose-, ribose-, nickel- and dipeptide-binding proteins, which have been implicated in chemosensing (Falke *et al* 1997). These proteins also serve as transport pathway components (Higgens 1992). In contrast to the periplasmic binding proteins, the MCPs are specialised for chemotaxis. There are four MCPs in *E. coli*, Tsr, which recognises serine; Tar, (aspartate and maltose) Trg, (ribose, glucose and galactose) and Tap, (dipeptides). Each chemoreceptor consists of two transmembrane helix (TM1, TM2), a periplasmic ligand binding domain and a cytoplasmic signalling domain (Figure 1.5a) and they are found clustered near the poles of the cell (Maddock and Shapiro 1993).

Communication between the receptors and the flagella switch involves four proteins: CheA, the histidine protein kinase; CheY, the response regulator; CheW, the receptor coupling factor and CheZ, an enhancer of CheY-P dephosphorylation. CheA and CheY constitute a prototype two-component system, although they deviate from the standard paradigm in several ways, most notably in that CheY does not contain a DNA binding domain nor acts as a transcription factor (Stock and Surette 1996). The *E. coli* CheY has been crystallised and the 3-dimensional structure determined (Figure 1.6) (Volz and Matsumara 1991). CheY is a doubly wound α/β with five α-helices surrounding a five-stranded parallel β-sheet (Fig. 1.6). The central strand, β_3, contributes D57, the acidic residue known to be phosphorylated in the activation process of CheY (Sanders *et al* 1989). The effects of binding to the ligand causes a conformational change in both domains of the chemoreceptor which are recognised by an associated CheA-CheW complex (Borkovich *et al* 1989, Gegner *et al* 1992). CheA has autokinase activity which is inhibited by attractant bound receptors and is stimulated by repellent or attractant free receptors. Stimulation of CheA causes it to donate its phosphate to an aspartate residue of the response regulator CheY which, in its phosphorylated form dissociates from the ternary complex and interacts directly with FliM in the flagellar switch complex to cause CW rotation

(Sanders *et al* 1989, Barak and Eisenbach 1992). CheZ counteracts the tumble signal by accelerating the decay of the CheY-P unstable aspartyl-phosphate residue (Blat and Eisenbach 1996). When an attractant binds to an MCP it results in reduced levels of CheA autophosphorylation, in turn less phosphate is transferred to CheY causing a switch from CW to CCW rotation. Thus, cells increase their run lengths as they enter areas of higher attractant concentration. If a repellent is present or there is no attractant the opposite effect occurs, resulting in increased CheY-P which interacts with FliM to cause CW rotation or tumbling (see Stock and Surette 1996, Falke *et al* 1997). As the bacterial cells migrate up or down chemical gradients, they must continually adapt to the prevailing concentrations of attractants and repellents. To accomplish this, adaptation is necessary.

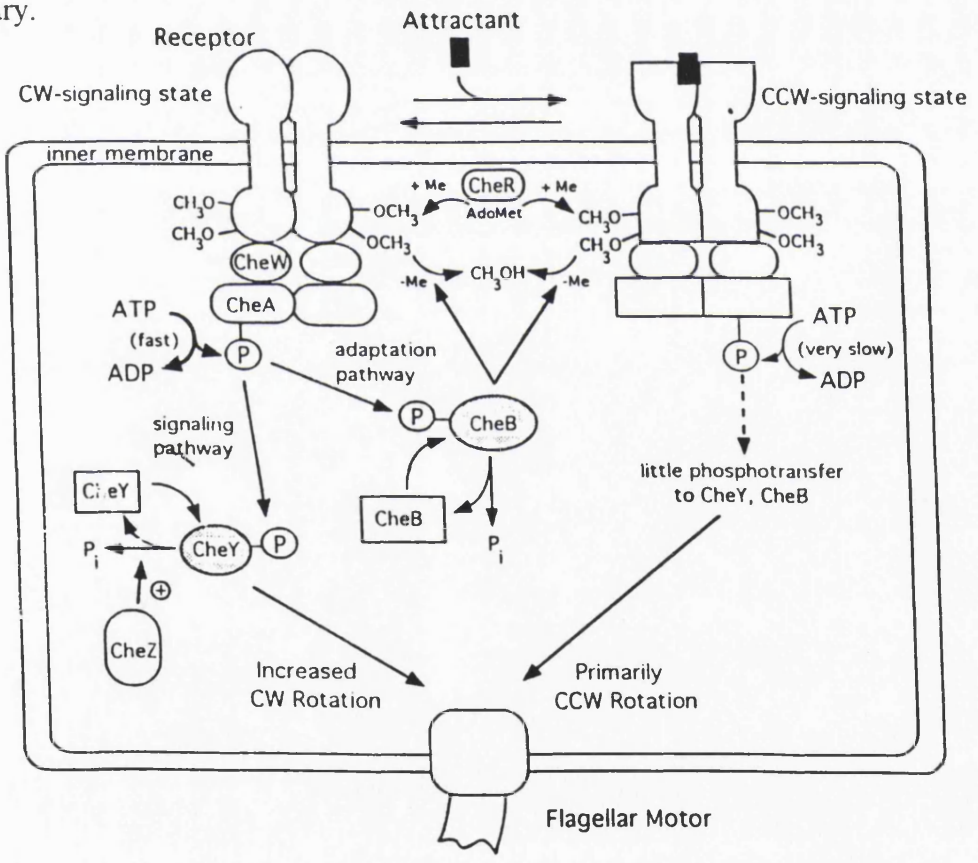


Figure 1.4: The chemotactic signalling and adaptation pathways of *E. coli* +Me signifies methylation and -Me demethylation. Notation explained further in text. Adapted from Blair (1995).

1.7.4 Adaptation by methylation

Adaptation is controlled by a feedback loop that covalently modifies 4-6 glutamate residues on the cytoplasmic surface of each receptor (Figure 1.5b). Two enzymes, the methyltransferase CheR and the methylesterase, CheB, are necessary for adaptive methylation. CheB is a target for phosphotransfer from CheA. In steady state, methyl addition by CheR balances methyl removal by CheB-P to achieve an intermediate level of methylation (0.5-1 methyl group per subunit) that maintains run-tumble behaviour (Kort *et al* 1975, Goy *et al* 1977). The binding of an attractant to the chemoreceptor causes a positive conformational change in the signalling domain resulting in a reduction in the autophosphorylation of CheA, causing lower levels of phospho-CheY and therefore straight swimming. This increase in attractant concentration also results in a reduced level of activated phospho-CheB. As the level of activated CheB-P drops the constant rate of CheR methyltransferase activity gradually increases the rate of methyl esters per receptor. Eventually the rising level of methyl esters stimulates histidine kinase activity, resetting the receptor signal to its basal level so that the new higher level of chemoattractant has been adapted to and is now seen as the normal level (Hazelbauer *et al* 1990, Blair *et al* 1995, Falke *et al* 1997)..

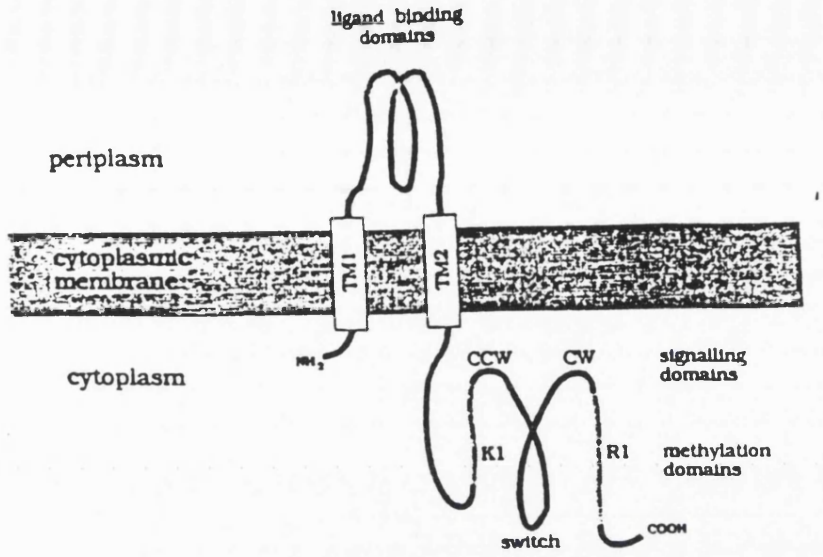
Loss of the chemoattractant from the receptor results in a negative conformational change in the signalling domain, causing autophosphorylation to be increased. This results in the phosphorylation of CheY causing CW rotation and tumbling. This also results in increased activation of CheB. The rising levels of CheB-P accelerates the removal of methyl groups such that it is back in the null state; this time the cell has adapted to a lower level of chemical (Hazelbauer *et al* 1990, Blair *et al* 1995, Falke *et al* 1997).

The receptor methylation level provides a simple chemical memory used to ascertain whether the current direction of swimming is favourable or

unfavourable. As the cell swims it compares the current environment, as monitored by the ligand occupancy of the receptor population, to the chemical environment of the recent past as 'remembered' by the methylation level. If the environment has significantly improved or deteriorated, the histidine kinase activity of the receptor kinase is inhibited or stimulated, respectively, thereby altering the probability of tumble events in the appropriate manner (Hazelbauer *et al* 1990).

A second putative adaptation mechanism in chemotaxis has been identified at the level of CheY-CheZ which involves oligomersiation dependant modulation of phosphatase activity. This hypothesis stems from observations that CheZ mutants adapt to attractants and repellents more slowly than the wildtype. CheZ is a dimer which undergoes oligomerisation upon interaction with CheY-P and this causes phosphatase activation of CheZ. These observations suggest that any modulation of the level of CheY phosphorylation by chemotactic stimuli may be counter-balanced by the rate limiting oligomerisation of CheZ. Therefore an increase in the level of CheY phosphorylation increases the CW bias of flagellar rotation. This in parallel causes oligomerisation of CheZ, which results in increased CheZ phosphate activity which dephosphorylates CheY-P, terminating the CW bias. Conversely, a decrease in the level of CheY-P decreases the CW bias of flagellar rotation. This causes a relatively slow dissociation of the CheZ oligomer, allowing levels of CheY-P to rise again. This proposed mechanism, in conjunction with methylation ensures that the phosphorylation level is set back to its pre stimulus level. This restores the unstimulated mode of swimming in the continued presence of a stimulus (Blat and Eisenbach 1996).

(a)



(b)

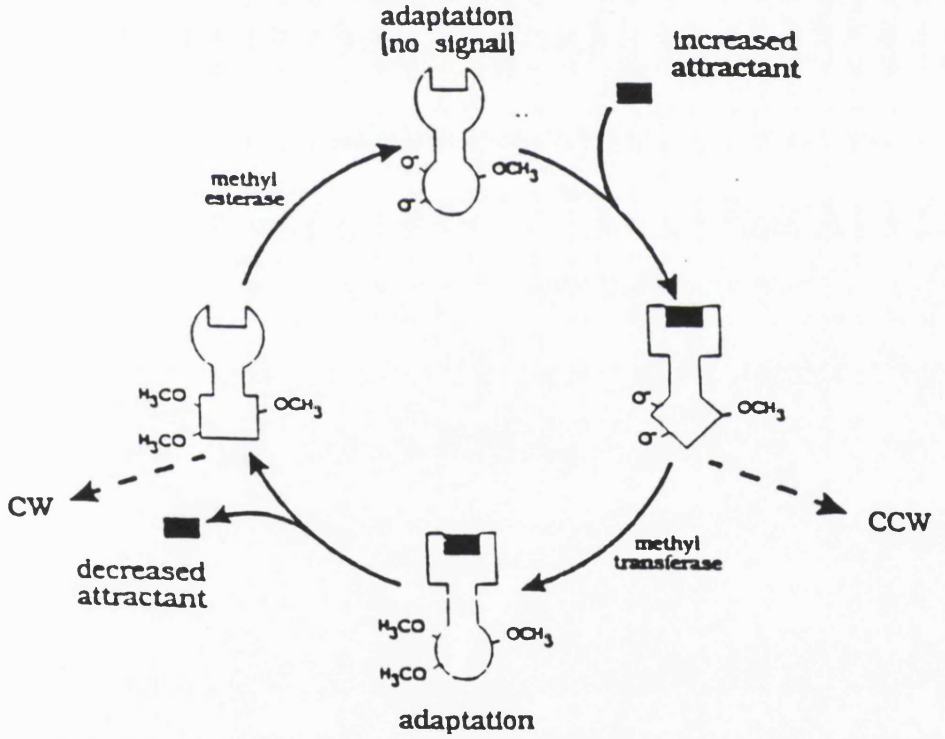


Figure 1.5: Structure and function of MCPs. (a) Structure of an MCP monomer showing different functional domains and (b) structural changes in MCP caused by ligand binding and subsequent methylation and adaptation. TM (transmembrane region). See text for detail. Adapted from Armitage (1992).

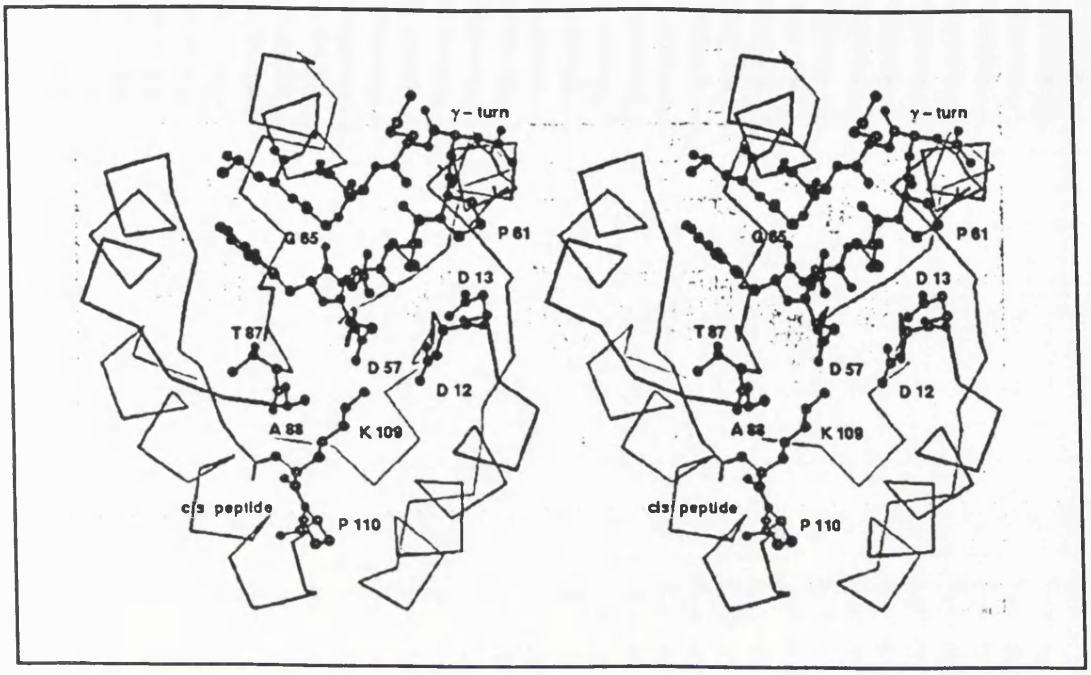


Figure 1.6: Stereo diagram of CheY region containing the most highly conserved residues of the family. P 61, G 65, T 87, T 88 and P 110 all play important roles in the maintenence of the conserved CheY structure (Volz 1993).

1.7.5 Methylation independent chemotaxis

In addition to responding to chemicals sensed by MCPs, bacteria also respond to a range of stimuli that do not interact with these dedicated sensory proteins. MCP-independent systems respond to primary growth limiting factors such as oxygen, light and some carbohydrates, and are therefore found associated with bacteria in the natural environment. In contrast the methylation-dependent system may be more important for directing "well-fed" bacteria to optimum environments (Armitage *et al* 1990). Two methylation-independent pathways found in *E. coli* include PT chemotaxis (chemotaxis to carbohydrates of the phosphoenolpyruvate phosphotransferase system or PTS) and aerotaxis (taxis towards or away from oxygen) (Postma *et al* 1993). The components involved in the PTS transport

system include an autophosphorylating histidine kinase, enzyme I (EI), a phosphocarrier HPr, phosphoenolpyruvate (PEP) and the enzyme II carbohydrate specific complexes (EII ABC). At the expense of PEP, EI autophosphorylates on a histidine residue and transfers this phosphoryl group to the histidine residue on HPr. HPr-P then energises the integral membrane EII ABC complex. IIC binds and transports the substrate into the cell during which it is phosphorylated by the IIB domain (Postma *et al* 1993).

Extensive genetic analysis of this pathway has established that both the methylation-dependant and independent chemotaxis pathways require the components CheA, CheW and CheY (Rowsell *et al* 1995). Further, it was found that unphosphorylated EI inhibits the autophosphorylation of CheA. This observation suggested that in response to the PTS chemotactic stimuli, during which EI accumulates, CheA is inhibited. This in turn lowers the level of CheY phosphorylation and the flagella are consequently biased more towards the CCW rotation or smooth swimming (Lux *et al* 1995). This model, if correct, shows interaction between the methylation-dependent and -independent signalling pathways.

The other methylation-independent pathway in *E. coli* is aerotaxis, or the response to oxygen. As with PT chemotaxis, aerotaxis also requires CheA, CheW and CheY (Rowsell *et al* 1995). An oxygen sensory transducer called Aer has been identified which may mediate aerotaxis by detecting changes in the redox state of a component of the electron transport chain. Aer has a carboxyl-terminal signalling domain similar to that of a classical MCP; it is through this that it may communicate with the chemotaxis signalling domain. In contrast the amino terminal domain is similar to the redox sensory proteins NifL and FixL (Bibikov *et al* 1997). Mutants lacking Aer lose positive aerotaxis but are still repelled by high levels of oxygen, indicating the presence of a second unknown receptor to mediate this phobic response (Rebbapragada *et al* 1997).

1.7.6 Other factors that affect signalling.

There are a number of small molecules that have been shown to have an effect on chemotaxis. One of these, fumarate can directly alter the motor switch in the archeon *Halobacterium salinarium* (Montrone *et al* 1996), an effect also seen in flagellated spheroplasts of *E. coli* (Barak *et al* 1995). The functional mechanism and role of fumarate remains obscure, but it may function by lowering the activation energy of switching. Fumarate could connect the bacterial metabolic state to chemotactic behaviour. Under starvation conditions, when the energy required for switching would be unavailable, fumarate may act to reduce the CW bias, so that the bacterium swims smoothly with few tumbles. This would be an efficient way to move the bacterium away from the location where they have insufficient energy and no directional information.

CheY can be phosphorylated *in vitro* by low molecular weight phosphodonors. One of them, acetyl phosphate, can be abundant *in vivo* depending on growth conditions. Its affect is not essential for chemotaxis, but it has been shown to influence the ability of the cells to swarm (McCleary and Stock 1994).

Another possible link between metabolism and chemotaxis may be the acetylation of CheY by the enzyme acetyl-CoA synthetase, with acetyl-CoA or acetate as acetyl donors. Acetylation causes a 4-5 fold increase in the CW-promoting activity of CheY in cytoplasmic free envelopes (Barak *et al* 1992). It is possible that under some conditions, acetylation might be involved in regulating CheY activity. Similarly addition of calcium to *E. coli* cells causes tumbling (Tisa and Adler 1992). The precise target of Ca^{2+} action has not been identified, but it requires CheA, CheW and CheY but not the MCP proteins. It has been proposed that Ca^{2+} helps to stabilise and maintain CheY in its phosphorylated state, thereby prolonging the tumble signal. This theory is supported by the fact that Ca^{2+} inhibits the dephosphorylation of CheY (Lukat *et al* 1990).

1.7.7 Co-ordinate regulation of chemotaxis and motility with virulence

To cause disease, successful pathogens have to survive a range of limiting environments including, low pH, stress and nutrient deprivation. After ingestion, bacteria adapt to the mammalian host temperature, a myriad of environmental assaults and the immune system. Motility and chemotaxis are essential virulence determinants for a number of pathogens, allowing them to reach the site of infection. However, the presence of flagella provides antigenic targets for the host immune system. As discussed earlier, phase and antigenic variation, has been reported for many bacteria including *H. pylori*, and this is one way in which the organism avoids the immune system. Another method involves the co-ordinate regulation of chemotaxis and motility with virulence. This rigorous control of expression ensures that once the site of colonisation is reached, motility is down regulated and other virulence factors are up regulated enhancing colonisation.

One recently published example of this type of system is the co-ordinate transcriptional regulation of flagellin genes (*fleB*) and the primary invasion factor (*invA*) in *Yersinia enterocolitica* (Badger and Miller 1998). For both *S. typhimurium* and *Bordetella bronchiseptica*, negative control of flagellar gene expression is crucial for events that occur during infection (Schmitt *et al* 1994). Mutagenesis of FlgM in *S. typhimurium* (anti-FliA protein) causes a severe defect in virulence (Schmitt *et al* 1994). In contrast, no decrease in virulence was caused by the inactivation of FliA, which encodes a flagellar-specific sigma factor required for motility (Schmitt *et al* 1996). It has been proposed that FliA may function in *S. typhimurium* by both positively and negatively regulating flagella gene expression (Schmitt *et al* 1996). In *B. pertussis*, virulence genes are co-ordinately regulated by a component system, BvgS (sensor) and BvgA (activator) (Weiss *et al* 1983, Arico *et al* 1989). When BvgAS is active, adhesins, toxins and several other potential virulence factors are expressed. In unfavourable conditions,

the BvgAS activity is eliminated and an entirely different set of genes, including flagella and motility loci are induced (Akerley *et al* 1992, Akerley and Miller 1993). A study by Scarlato *et al* (1991) demonstrated that adhesion and toxin avoidance of the host immune system are two steps separate in time at the molecular level.

Studies of *Vibrio cholerae* have also identified interesting links between expression of virulence determinants and motility. Mutations in *V. cholerae* motility phenotypes produced increased amounts of toxin and toxin-regulated pili (TCP), whilst hypermotile mutants of *V. cholerae* were shown to be defective in the production of toxin and TCP (Gardel and Mekalanous 1996). Mutagenesis of *acfB* (homologous to methly-accepting chemotaxis protein) or *tcpI* (encodes TlpA, the major pillin subunit of TCP) increase motility, suggesting that AcfB and TcpI promote colonisation by limiting bacterial movement (Harkey *et al* 1994, Everiss *et al* 1994). Additionally it has been demonstrated that chemotaxis must be down regulated in order for binding to occur (Gardel and Mekalanous 1996).

Finally it has been proposed that a similar mechanism exists in *C. jejuni*. Non chemotactic mutants show a threefold increase in binding and invasion *in vitro*, while a diploid *cheY* strain has a reduced ability to bind and invade (Yao *et al* 1997). This suggests that chemotaxis must be down regulated for the course of bacterial infection to proceed.

1.7.8 Range of chemotaxis systems.

The principles of the chemotaxis system are best understood in *E. coli* and *S. typhimurium*. However the chemosensory system has been adapted in different bacterial subgroups in a way which may relate to the different environmental niches into which they evolved. All systems retain the two component regulatory system of CheA and CheY. Differences between the systems include the number

of homologues of each *che* gene, the effects of CheY-P on the switch, the mechanism of adaptation and the number of signal pathways. The main characteristics of the chemotaxis systems discussed in this chapter are summarised in Table 1.3.

Homologues of the main chemotaxis proteins of *E. coli*, CheA, CheB, CheR, CheW and CheY are also present in the gram positive bacterium *B. subtilis*, but play different roles in the two organisms. CheA still phosphorylates CheY in response to signals from the MCPs, causing CheY-P to interact with the flagellar motor. However, increased levels of CheY-P have the opposite effect in *B. subtilis*, inducing CCW rotation of flagella resulting in smooth swimming behaviour (Bischoff *et al* 1993). There are also differences in the methylation and adaptation systems of *B. subtilis*. For example, both positive and negative stimuli induce methyl groups to be transferred to a regulator throughout the adaptation period (Kirby *et al* 1997). In contrast, in *E. coli*, positive stimuli cause increased methylation of MCPs, while negative stimuli cause decreased MCP methylation (Goy *et al* 1977). Additionally all stimuli cause the formation of enhanced methanol production in *B. subtilis* (Thoelke *et al* 1989), whilst negative stimuli increase methanol production and positive stimuli suppress methanol production in *E. coli* (Toews *et al* 1979). Work by Toews *et al* (1979) also demonstrated that it was the rate of transfer of the methyl groups to the regulator that brings about the chemotactic response in *B. subtilis*, in contrast to the system in *E. coli* where the response is dependent on the absolute level of MCP methylation. In *B. subtilis*, CheB or CheR null mutants exhibit partial adaptation to some attractant stimuli, suggesting the presence of a methylation-independent adaptation system. Recent work by Garrity *et al* (1998) has proposed the existence of a PTS system controlling chemotaxis to carbohydrates which also requires an MCP component to function. The *B. subtilis* chemotactic operon also contains two novel proteins, CheC and CheD, which have been shown to interact and affect methylation of

MCPs (Rosario and Ordel 1996). CheC functions to inhibit binding of CheD to MCPs. CheD is necessary for normal methylation and basal activation of CheA (Rosario *et al* 1995). It has been proposed that in the presence of high levels of CheY-P, CheC is activated to interact with CheD, preventing it binding to MCPs. This in turn results in the inhibition of CheA and hence a reduction in levels of CheY-P (Rosario and Ordal 1996).

A further complication in the *B. subtilis* chemotaxis system is the presence of a novel chemotaxis gene CheV, which contains an amino terminal CheW homologous domain linked to a response regulator domain of the CheY family (Fredrick and Helman 1994). A CheV mutant strain is motile but impaired in chemotaxis on swarm plates. An insertion mutation in the CheY domain displays only a modest defect in chemotaxis. Previous studies of *B. subtilis* chemotaxis genes had indicated that CheW null mutants were still capable of reduced chemotactic signalling (Hanlon *et al* 1992) suggesting that CheW did not function in *B. subtilis* as it did in *E. coli.* However, the finding of a second CheW homologous domain, and the observation that strains containing a deletion of both *cheV* and *cheW* failed to chemotax confirmed that CheW function was essential (Rosario *et al* 1995). Why both proteins are required for optimum chemotaxis is not fully understood; they may interact with different subsets of MCPs or be active at different times. The role played by the second CheY homologue has yet not been elucidated. However, if CheV is coupling MCPs to CheA, it seems unlikely that CheY can also interact with the switch. The CheY homologue may function to recruit the CheA kinase to the MCPs, serve as a competitive inhibitor of CheY phosphorylation or function as a substrate for another protein kinase.

Factors/Species	*E. coli*	*B. subtilis*	*S. meliloti*	*R. sphaerodis*	*R. centenum*	*H. salinarium*	*M. xanthus*	*H. pylori*
Flagella	√	√	√	single flagellum	on solid media only	√	x	√
CW	SS	T	SS	CW swimming only	SS	Forward swimming	unknown	unknown
CCW	T	SS	T		T	Reverse swimming	unknown	unknown
No. of CheY's	1	2	2	3	2	1	3	This study
No. of pathways	1	1	1	2		1	1	This study
Novel proteins	unknown	CheC, D, V	CheD	CheD	CheAY	unknown	CheAY/FreZ CheYY/FrzB	Proteins absent CheB, R, Z
MCP's: classical	√	√	√	unknown	unknown	√	√	√
Tlp's	unknown	unknown	√	√	unknown	√	unknown	unknown
soluble	unknown	unknown	unknown	unknown	unknown	√	unknown	√
Methylation dependant	√	√	√	√	unknown	√	unknown	unknown
Methylation independant	√	√	unknown	unknown	unknown	unknown	unknown	

Table 1.3: The main features of the range of bacterial chemotaxis systems detailed in sections 1.7.3 - 1.7.8.
SS - Smooth swimming, T - tumbling.

Another example of a chemotaxis system containing multiple *che* homologues is found in *Sinorhizobium meliloti*. *S. meliloti* is a Gram negative soil bacterium which is a member of the α subgroup of bacteria. It is an obligate aerobe, capable of nitrogen fixation and has a number of peritrichously located flagella which come together to form a unidirectional CW rotating bundle (Gotz and Schmitt 1987). The flagella rotate with no intermittent stops, but with considerable fluctuations in rotary speed. Direct observation of swimming cells by video enhanced DIC microscopy showed that two swimming modes were seen: rapid forward swimming with the flagella forming rotating bundles, and directional changes where individual flagella rotate at different speeds driving the flagellar bundle apart (Armitage and Schmitt 1997).

The *che* operon found in *S. meliloti* encodes homologues of the central components of the *E. coli* signal transduction chain, CheW and CheA, as well as homologues of *E. coli* CheR and CheB, responsible for adaptation, and two response regulators CheY1 and CheY2 (Greck *et al* 1995). The addition of the strong attractant proline causes a sustained increase in swimming speed (chemokinesis) and increases the smooth swimming rate, resembling the suppression of tumbles seen in enterobacteria (Sourjik and Schmitt 1996). In *S. meliloti* the addition of attractant causes inhibition of CheA autokinase and removal causes activation with the consequent production of CheY1-P and CheY2-P which act to slow the flagellar motor (Sourjik and Schmitt 1996). Mutational studies with both CheY1 and CheY2 suggest that CheY2 is the chief regulator of chemotaxis and that CheY1 may play the role of the absent CheZ, by acting as a competitor of CheY2 for phosphorylation by CheA (Sourjik and Schmitt 1996).

S. meliloti has at least 4 MCPs in addition to two transducer-like proteins, TlpA and TlpB (Armitage and Schmitt 1997, Greck *et al* 1995). Both TlpA and TlpB contain a conserved signalling domain, but neither a hydrophobic transmembrane

domain nor a periplasmic receptor domain, both of which are features of classical MCPs (Greck *et al* 1995). TlpA and TlpB are thought to be cytoplasmic transducers with roles in sensing internal stimuli that reflect the metabolic state of the cell. Mutants in *tlpA* and *tlpB* swim with reduced speed, possibly due to differences in the extent to which CheA autokinase activity is inhibited thereby preventing CheY phosphorylation and resulting in an increased flagellar rotation rate (Armitage and Schmitt 1997). The adaptation process in *S. meliloti* has yet to be explained, however, analysis to date suggests that it is more similar to that found in *B. subtilis* than *E. coli*. The finding of an ORF in the *che* operon homologous to *B. subtilis* CheD supports this view (Armitage and Schmitt 1997).

Extensive research of the related bacterium *Rhodobacter sphaeroides*, also a member of the α subgroup, reveals a more complex system containing multiple copies of many *che* genes. *R. sphaeroides* is a purple non-sulphur bacterium which can grow aerobically as a heterotroph or anaerobically as a photoheterotroph. *R. sphaeroides* has a single medially located flagellum, which rotates clockwise (Armitage and Macnab 1987). Occasionally variants have been identified with a CCW flagellar motor, though in neither case have motors been seen to switch (Packer and Armitage 1994). The bacterium swims and stops, re-orientating during a stop. Examination of the flagellum during this stop found that the flagella relaxes from the distal end to form a short wavelength high amplitude coil against the cell body. The coiled flagellum can still slowly rotate and this contributes to the re-orientation of the bacterium. The helix then reforms, from the cell body out, and the cell swims off in a new direction (Armitage and Macnab 1987).

The predominant chemoattractants in *R. sphaeroides* are weak organic acids which cause a short term change in the stopping frequency and in the duration of stops and runs, eventually leading to accumulation (Poole and Armitage 1989). This response is described as "pessimistic" with cells sensing a reduction in

attractants, in contrast to *E. coli*, which responds primarily to the increase in attractant concentration. The behaviour of aerobically grown cells is more complex and probably reflects the more complex sensory pathways induced under these conditions. *R. sphaerodis* also exhibits an independent chemokinetic response, characterised by a sustained increase in swimming speed which is thought to lead to dispersal (Packer and Armitage 1994). In contrast to *E. coli*, taxis to chemoattractants in *R. sphaerodis* has been shown to require transport and partial metabolism (Ingham and Armitage 1987, Poole and Armitage 1989 and Jeziore-Sassoon *et al* 1997). As with *S. meliloti*, *R. sphaerodis* lacks the conventional MCPs, but instead contains two transducer-like proteins TlpA and TlpB (Ward *et al* 1995a). As the Tlp proteins lack putative sensory domains, they are thought to sense specific cytoplasmic intermediates, although the mechanism has not yet been elucidated. Mutagenesis of *tlpA* results in a reduction in chemotaxis, but only under aerobic conditions (Ward *et al* 1995a). *R. sphaerodis* has been shown to use different pathways for the same metabolites under aerobic and anaerobic conditions (Hamblin *et al* 1997). This contrasts to *E. coli*, where each MCP is responsible for dealing with a subset of chemoeffectors. The process of methylation and adaptation has not yet been elucidated for *R. sphaerodis*, but preliminary work (Manson *et al* 1998) suggests that CheR, CheB and the CheD like ORF found in *R. sphaerodis* may constitute a methylation system more closely related to that observed in *B. subtilis* than *E. coli* (Armitage and Schmitt 1997).

In *R. sphaerodis*, deletion of the chemotaxis operon containing the genes encoding CheA, CheW, CheR, CheY and CheY2, resulted in no major phenotypic change (Ward *et al* 1995b). This was explained by the identification of a second chemotaxis operon which contained a second CheA, a third copy of CheY, two additional CheWs and the only copy of CheB identified to date. Deletion of CheA2 resulted in the loss of chemotaxis and altered phototaxis (Hamblin *et al*

1997). It is not known how these pathways are integrated and preliminary data suggests the presence of a third copy of CheA, which when deleted results in the complete loss of chemotaxis and phototaxis (Armitage and Schmitt 1997). Interestingly the mutant cells still swim normally, not exhibiting any extreme smooth or tumbly phenotypes, suggesting that some aspects of motor control may be independent of the chemosensory pathway.

Flagellar rotation is driven by the proton motive force (Δp) across the membrane. In *E. coli*, under conditions of saturated Δp, there is little change in the rotation rate of the flagella (Armitage and Schmitt 1997). In contrast both *R. sphaerodis* and *S. meliloti* show major changes in the rate of flagellar rotation under such conditions, suggesting that they have a mechanism for "uncoupling" the rotation rate from the proton motive force (Poole *et al* 1990, Brown *et al* 1993). Both *R. sphaerodis* and *S. meliloti* contain chemotaxis systems that are more associated with a soil/water type environment than that found in *E. coli*. The integration of chemosensory pathways dependent on the growth state may be more suited to the fluctuating environment of soil and water bacteria. Additionally the presence of multiple chemotaxis homologues may promote survival of *R. sphaerodis* and *S. meliloti* in different environments.

Another member of the α subgroup, *Rhodospirullium centenum* has yet another combination of chemotaxis homologues which are integrated into a slightly different chemotaxis system. *R. centenum* is a purple non-sulphur photosynthetic bacterium. When grown in liquid medium *R. centenum* cells are seen to tumble or reverse direction on migration into a darker region, a phenomenon termed the scotophobic response (Gest *et al* 1995, Ragatz *et al* 1994). As in *E. coli*, CCW rotation of the polar flagellum leads to smooth swimming and CW rotation leads to either tumbling or reversal of cell movement. However when *R. centenum* is grown on solidified media numerous peritrichous flagella are produced and the bacteria migrate towards the light source in a process called phototaxis (Hader

1987), which is similar to the trial and error process of 'walking up' a chemical gradient. Identification of a chemotaxis operon found genes encoding homologues of CheW, CheY, CheB and CheR (Jiang and Bauer 1997). Additionally an ORF coding for a CheA-CheY hybrid polypeptide was identified. Mutants in *cheB*, *cheR* and *cheW* which give rise to autophosphorylated CheY or CheAY, resulted in a smooth-swimming phenotype. This was also observed for disruption of CheAY. Therefore, the chemotaxis signal transduction cascade appears similar to that of *E. coli*. Disruption of *cheY* results only in a slight increase in frequency in liquid grown swimmer cells and has no effect on chemotaxis. In contrast a complete lack of surface motility is observed in *cheY* mutant swarm cells (Jiang *et al* 1997). It may be that CheY is involved in controlling lateral flagellar rotation, whereas CheAY is involved in controlling polar rotation. This theory is further supported by the finding that the polar and lateral flagella are composed of different flagellin and basal ring subunits, and that the residues required for docking to FliM and FliG (Sockett *et al* 1992) are not conserved between CheAY and CheY. The two CheY homologues found in *R. centenum* may play the same role played by the two CheY proteins found in *S. meliloti* (Sourjik and Schmitt 1996), with the CheY domain of CheAY acting as the main response regulator, whilst the second CheY functions as a phosphate sink.

Both positive and negative phototactic responses were abolished in all chemotaxis mutants indicating that eubacterial photosensory perception involves transmission of a signal to the flagellar motor via the chemotaxis sensory transduction cascade (Jiang *et al* 1997). Whether chemotaxis and phototaxis are controlled through one pathway, or whether two converging pathways control light and signal transduction is unknown. In contrast to the system in *E. coli* where swarmer cell differentiation is abolished by defects in the *che* genes (Harshey 1994), *R. centenum* swarming differentiation appears to be similar to that of *V. parahaemolyticus*, which is known to undergo swarming differentiation

independently of a functioning chemotactic signalling cascade (McCarter and Silverman 1990).

Chemotaxis has also been studied in a member of the archaea, *Halobacterium salinarium* . This organism is capable of both chemo- and phototaxis (Oesterhelt and Marwan 1990). *H. salinarium* has polar bundles of 5-10 flagella (Marwan *et al* 1991) each of which is driven by an individual motor. Both rotation and switching of individual filaments is synchronous. In the absence of stimuli, *Halobacterium* performs a random walk based on the spontaneous switching of the flagellar motor from CW (forward swimming) to CCW (reverse swimming) (Alam and Oesterhelt 1984), with cells spending equal time swimming forwards and backwards.

Genes encoding the *che* homologue, CheA, CheY and CheB have been cloned from *H. salinarium* . This was the first two-component response regulator system identified in the kingdom archaea (Rudolph and Oesterhelt 1995, Rudolph *et al* 1995). The loss of both chemo- and phototaxis upon disruption of *cheA*, indicates that CheA is a component of both the light and chemical signal transduction pathway. In contrast to *E. coli*, the *H. salinarium cheA* mutant performs straight swimming regardless of the direction of the flagellar motor (Rudolph *et al* 1995). This implies that in *H. salinarium*, CheY-P promotes the switching event from CCW to CW or visa versa, rather than mediating CW rotation specifically, as is the case in *E. coli*. An additional difference between the *Halobacterium* and *E. coli* response lies in the adaptation response mediated by CheB. In *H. salinarium*, like *B. subtilis*, both negative and positive chemotaxis cause an increase in methanol release during adaptation (Rudolph *et al* 1995). Methanol release is only observed in *E. coli* during adaptation to negative stimuli (Kehry *et al* 1984).

H. salinarium has a total of 13 transducer proteins, either soluble or membrane bound (Zhang *et al* 1996). They fall into three subfamilies: some resemble bacterial chemoreceptors, with large periplasmic and cytoplasmic domains and

two membrane spanning helices, whereas some contain multiple amino-terminal transmembrane segments but lack a periplasmic domain; the third group is characterised by soluble cytoplasmic proteins (Zhang *et al* 1996).

The bacterium *Myxococcus xanthus* is a gram negative rod shaped bacterium which moves across surfaces by gliding motility (Zusman *et al* 1990). This bacterium displays both chemokinetic and chemotactic behavioural responses (Dworkin and Eide 1983, Shi *et al* 1993) and individual *M. xanthus* cells move up chemical gradients (Tieman *et al* 1996). However, under nutrient rich conditions, groups of vegetative cells colonise new areas by moving as a communal swarm, releasing hydrolytic enzymes to digest nutrients. The motility system of *M. xanthus* is divided into two parts, A (adventurous) and S (social) motility. A motility is more apparent on dry or solid surfaces and allows individual cells to move in the absence of others. Conversely, S motility occurs under moist conditions and is thought to be involved in the formation of fruiting bodies (Hartzell and Youderian 1995).

The ability of cells to translocate directionally over solid surfaces is regulated by the Frz signal transduction system (Zusman and M^{c}Bride 1991). The components of this system are clustered on the chromosome and are homologous to the *che* genes from enteric bacteria (M^{c}Bride *et al* 1989). FrzA, FrzF, FrzG and FrzCD are homologues of CheW, CheR, CheB and an MCP respectively. FrzE consists of an N-terminal CheA domain joined to a C-terminal CheY domain (similar to the bifunctional CheAY found in *S. meliloti*), and FrzZ is composed of two CheYs. FrzB has no enteric homologue. The phenotypes of there mutants are also reminiscent of enteric bacteria (Blackhart and Zusman 1985). However, there are also substantial differences between the chemotaxis systems of *M. xanthus* and *E. coli*. *E. coli* are motile in liquid media and control their movements in three dimensions by random redirection during tumbling. *M. xanthus* can only move on a solid substrate in two dimensions, and redirection occurs primarily because of

inconsistencies on the surface on which the cells glide. Another striking difference is the speed and time scales with which the bacterium swim and adapt. *E. coli* moves at a rate of about 1500 μm/min, changing direction every 1-2 secs. In contrast *M. xanthus* cells move at about 2 μm/min changing direction every 7-8 mins (Zusman et al 1990). Finally, only responses of *M. xanthus* to repellent stimuli are similar to responses of enteric bacteria. Responses to attractant stimuli appear more complex, involving all of the *che* homologues and FrzB (Tieman *et al* 1996).

1.7.9 Chemotaxis in *H. pylori*

H. pylori resides mainly in the surface mucous layer in the stomachs of infected subjects (Ogata *et al* 1998, Kirschner and Blaser 1995). As the gel layer has a rapid turnover (Kirschner and Blaser 1995), bacteria proliferating in the mucous layer must have the ability to move toward the epithelial cell surface, against the mucous flow toward the duodenum. Thus chemotaxis by flagellar locomotion is likely to be important in colonisation by *H. pylori.* Very little is known, however, about the process of chemotaxis in this bacterium.

Some chemoattractants have been identified for *H. pylori* including mucin (Turner *et al* 1997). Mucin, a glycoprotein of high molecular weight is the principle constituent of mucus, and is secreted from epithelial cells of intestinal, gastric and gall bladder tissues (Ogata *et al* 1998). A recent study by Mizote *et al* (1997) demonstrated a chemotactic response by *H. pylori* wildtype and *ureB* mutant strains to urea and flurofamide (a potent urease inhibitor and non-metabolisable structural analogue of urea) and sodium bicarbonate. This response is increased in a high viscosity environment, a condition that mimics the ecological niche of *H. pylori* (Nakamura *et al* 1998). Bicarbonate and sodium are secreted into the gastric mucosa near the parietal cells and surface mucosa cells, respectively (Stuart-Tilley *et al* 1994). Urea is supplied by the capillary networks beneath the

basement membrane of epithelial cells and a concentration gradient is thought to be formed in the mucus layer (Neithercut *et al* 1993). The epithelial cell surface location of both sodium and urea may guide the bacteria to this site. Such chemotaxis may also be important nutritionally as ammonia (produced by urease activity from urea) can serve as a nitrogen source for *H. pylori* growth (Mizote *et al* 1997).

Treatment of bacterial cells with the urease inhibitor acetohydroxamic acid prior to chemotaxis assay resulted in a complete loss of chemotactic activity in response to sodium bicarbonate in addition to partial loss of urease activity. In contrast, the action of the inhibitor flurofamide on the both the chemotactic and urease activities was partial. These results suggest that cytoplasmic urease is more important that surface urease for the chemotactic motility of *H. pylori*, as acetohydroxamic acid is a small molecule capable of penetrating intact bacterial cells. Flurofamide is a non diffusable inhibitor and does not therefore inhibit cytoplasmic urease (Nakamura *et al* 1998). These authors proposed that intracellular urea hydrolysed by cytoplasmic urease may supply the proton motive force required to drive the bacterial flagellar motor, and *H. pylori* chemotaxis toward urea may serve to provide urea to be hydrolysed externally for gastric acid neutralisation by surface urease (Nakamura *et al* 1998).

Little is known about the molecular mechanism of chemotaxis in *H. pylori*, although a CheY homologue has been identified recently on a "stress responsive operon" (sro) which is co-ordinately expressed with the genes located downstream, including the cell division protein, FtsH, and the heavy metal binding proteins, CopA and CopP (Beier *et al* 1997). The annotated genome sequence of *H. pylori* strain 26695 contains nine putative chemotaxis homologues, CheAY (HP0392), CheW (HP0391), three CheV's (HP0393, HP0019, HP0616), CheY1 (HP1067) and three putative classical MCP's (HP0099, HP0082, HP0103) (Tomb *et al* 1997). Additionally, the gene encoding a truncated

soluble MCP homologue (with amino acid sequence similarity to MCPs restricted to the highly conserved domain, HCD) was isolated and its structure analysed (Allan 1997) (HP0599). Finally, analysis of the annotated genome has identified over 40 genes which based on homology to characterised *E. coli* and *S. typhimurium* proteins, are likely to be involved in the biosynthesis and export of flagella. The putative chemotaxis and motility genes identified in the annotated genome of *H. pylori* represent a large proportion of the relatively small genome. This stresses the importance that chemotaxis and motility play in the organisms survival, colonisation and persistence.

1.8 Aims and Objectives

H. pylori colonises the mucous layer of the gastric epithelium and is thought to cause gastritis, peptic ulcer disease, gastric adenocarcinoma and gastric lymphona. Therefore, it is important to understand the mechanism by which *H. pylori* colonises and persists in the mucous layer of the human stomach. Chemotaxis is a vital adaptation for many bacteria capable of colonising mucosal surfaces.

Therefore the aims of this study were:

(1) To amplify chemotaxis homologues from *H. pylori*

(2) To construct defined isogenic mutants in the chemotaxis homologues

(3) To identify the chemotaxis expressed gene products by 2D SDS PAGE gels

(4) To characterise by *in vitro* analysis the function of these genes in the chemotaxis and motility of *H. pylori*

(5) To compare the *H. pylori* wildtype and chemotaxis mutant strains ability to colonise both the gnotobiotic piglet and mouse models of infection

Chapter 2

Materials and Methods

2.1 Bacterial Strains and Growth Conditions

H. pylori strains used in this study where obtained from a variety of sources as outlined in Table 2.1. *H. pylori* were cultured on Blood Agar Base containing 7% v/v lysed horse blood (TCS Microbiology, Buckingham, UK) and Dents selective supplement (Oxoid, Basingstoke, UK) final concentrations: vancomycin (10 μg/ml), colomycin (10 U/ml), trimethoprin (5 μg/ml) and amphotericin B (5 μg/ml). Agar plates were incubated microaerophilically in a variable atmosphere incubator (VAIN, Don Whitley Scientific Ltd) at 37°C in an atmosphere of 7% CO_2, 5% O_2, 2% H_2 in N_2 for up to 2-3 days. All *E. coli* strains (Table 2.1) were grown in Luria broth or agar at 37°C. When necessary media was supplemented with either ampicillan (100 μg/ml), kanamycin (50 μg/ml), chloramphenicol (25 μg/ml) or tetracycline (15 μg/ml). 5-bromo-4-chloro-3-indoyl-B-D-galactoside (X-gal) (200 μg/ml) and isopropylthio-B-D-galatoside (IPTG) (50 μg/ml) were added as the majority of cloning experiments utilised the vector pUC19 which contains the *lac*Z DNA sequence (Sambrook *et al* 1989).

Table 2.1 Bacterial Strains

Bacterium		Source
H. pylori		
NCTC 11637		PHLS Collindale
N6		Dr. Ferrero
26695		Dr. Eaton
SS1		Dr. Ferrero
E. coli		
XL1-Blue MRF'	Δ(mcrA)183, F', Tn10 (tet^r)	Stratagene, Cambridge
XL2-Blue MRF'	F', cam^r	Stratagene, Cambridge
XLOLR	Δ(mcrA)183, F', Tn10 (tet^r), Su^-	Stratagene, Cambridge

2.1.1 Sources of chemicals and reagents

Chemicals were obtained from Sigma Chemical Co. (Poole, UK) unless stated otherwise, and were technical or reagent grade. Solvents were obtained from BDH (Poole, UK).

Plasmid	Description
pUC19	High copy number vector
pBK-CMV	Phagmid rescued from ZAP™ Express lambda vector
pJMK30	*C. coli* 1.4 kb *apha*-3 gene
pILL575	Cosmid Shuttle vector
pCAT	*C. coli* 0.8 kb *cat*GC gene
pCY	pUC19 containing 330 bp RR1
18B	pBK-CMV plasmid containing putative 5 kb *cheY*1 insert
18C	pBK-CMV plasmid containing putative 3 kb *cheY*1 insert
pCY110	pUC19 containing 1 kb insert with total *cheY*1
pCYIP	pCY110 disrupted by IPCRM
pCYIPK1	*apha*-3 gene inserted into the *Bgl*II site of pCYIP, opposite orientation
pCYIPK2	*apha*-3 gene inserted into the *Bgl*II site of pCYIP, forward orientation
pILLCA	pILL57 containing 6 kb DNA fragment with putative *cheA*
pCA110	pUC19 containing 0.7 kb *cheA* gene fragment
pCAIP	pCA110 disrupted by IPCRM
pCAIPK	*apha*-3 gene inserted into the *Bgl*II site of pCAIP, forward orientation
pCAIPc	cat_{GC} gene inserted into the *Bgl*II site of pCAIP
pCF	pUC19 containing 300 bp *cheY*
pCFIP	pCF disrupted by IPCRM
pCFIPK	*apha*-3 gene inserted into the *Bgl*II site of pCFIP

Figure 2.1a: List of plasmids used and constructed during this study

2.2 General Molecular Biology Techniques

2.2.1 Preparation of Plasmid DNA from *E. coli* Host Cells.

Preparation of plasmid DNA was carried out using two kits supplied by Promega. The Miniprep DNA Purification system was used for preparation of smaller quantities of plasmid and for purer samples for subsequent cycle sequencing. For larger yields of plasmids, Maxiprep kits were used to give yields of 1-2 mg. All buffers, columns and protocols were supplied by Promega Ltd., Southampton, UK.

2.2.2 Restriction Analysis of Genomic and Plasmid DNA

Restriction analysis of genomic and plasmid DNA was performed using restriction enzymes and buffers supplied by Promega. The manufacturers instructions were followed with regard to choice of optimal buffer conditions and reaction temperatures. 1U of enzyme was routinely used to digest 1μg of DNA in 1 hr. Following digestion, DNA to be used in further enzymactic reactions was purified using DNA Wizard claen-up kit (Promega) following manufacturers instructions.

2.2.3 Modifications of plasmid DNA and PCR products

T4 polymerase and Mung bean nuclease were used respectively to generate PCR products and digested plasmid DNA with blunt ends. Reactions were carried out according to manufacturers instructions. Both enzymes were supplied by Promega.

2.2.4 Ligation of Plasmid DNA

Reactions were carried out using T4 DNA Ligase and Ligase buffer supplied by Promega. All reactions were incubated overnight at 16°C. Typically the molar concentration of the insert was 2-3 times the concentration of the vector.

2.2.5 Agarose gel electrophoresis

DNA samples were run on 0.8 - 1.5% agarose gels made up in 1X TAE Buffer (40 mM Tris-acetate, 1 mM EDTA [pH8.0]) containing 0.5 μg/ml ethidium bromide. The gel concentration depended on the size of the DNA fragments been resolved. Electrophoresis was carried out at a constant 100 V and the DNA was visualised under a UV transilluminator (Model Tm-20, UVP Ltd., Cambridge, UK). A 1 kb molecular size ladder (Gibco-BRL Ltd., Paisley, UK) was run on each gel as a marker.

2.2.6 Purification of DNA Fragments from Low melting point agarose

Low melting point agarose gels (1%; SeaPlaque GTG agarose, Flowgen Instruments Ltd., Sittingbourne, UK) prepared in TAE buffer (40 mM Tris-acetate, 0.1 mM EDTA [pH 8.0]) were used to extract linear fragments for use in cloning experiments and as DNA probes in hybridisation experiments. To minimise damage to DNA the gels were post-stained with 0.5 μg/ml ethidium-bromide for 30 mins after electrophoresis and a long wavelength UV light source (Model UVM-57, UVP Ltd.) was used to visualise the DNA. DNA fragments were excised as gel slices and those to be used in subsequent cloning experiments were purified using the DNA Wizard clean-up kit (Promega) following the manufacturers protocol.

2.2.7 Transformation of *E. coli*

Competent *E. coli* XL2-Blue MRF' and XL1-Blue MRF' were purchased from Stratagene (Cambridge, UK). Transformation procedure was carried out as recommended by the manufacturer.

2.2.8 DNA Amplification using the Polymerase Chain Reaction (PCR)

A standard PCR mixture consisted of 0.01-100 ng of template DNA, 0.1-0.2 nmoles of each primer, 0.2 mM of each deoxynucleotide triphosphate and 1-2 units of *Taq* DNA polymerase (Appligene, Co. Durham, UK) in a total volume of 20-50 µl of 1X reaction buffer (10 mM Tris-HCL, 50 mM KCL, 1.5 mM $MgCl_2$, 0.1 % TritonX 100, 0.2 mg/ml BSA, [pH 9.0]). 50 µl of mineral oil (Sigma, RNase, DNase and protease free) was added to each tube to prevent evaporation of the PCR reaction mixture during thermal cycling. Table 2.4 contains the oligonucleotides used for PCR. Conditions for PCR cycling were adapted from those described by Sambrook *et al* (1989) and consisted of the following stages.

Table 2.2 PCR stages

Stage	No.	Temp (°C)	Cycle Time	Cycle No.
Stage 1	1	94	2.00	1
Stage 2	1	94	1.00	
	2	A	1.00	X
	3	B	C	
Stage 3	1	72	7.00	
	3	25	Hold	

Temperature A and B (°C) and time C (min) were adjusted according to individual PCR requirements. PCR reactions carried out included PCR with degenerate primers, to isolate novel genes, Inverse PCR Mutagenesis, to generate deleted plasmid constructs for the generation of mutants, specific primer PCR, to amplify DNA of known sequence and vector primer PCR, which was used to size

select putative clones. A summary of the different conditions typically used for the PCR reactions carried out are listed below.

Table 2.3 PCR cycling conditions

Type	Annealing Temp (A)	Time	Cycles (X)	Extension Temp(B)	Time
PCRDOP	30-45	1	30-40	72	1
IPCRM	45-55	1	40	72	3-5
SPPCR	40-55	1	30-40	72	1-4
VPPCR	50	1	30	72	1-4

2.2.9 DNA Sequencing

Sequencing of DNA was performed using a PRISM^{TM}dyedexoy terminator cycle sequencing kit (Applied Biosystems, Warrington, UK). Template of high purity was prepared from 10 ml of an overnight *E. coli* culture as described in section 2.2.1. PCR cycle sequencing reactions were performed following protocols described by the manufacturers and were analysed using an ABI373A automated sequencer (Applied Biosystems). Table 2.4 contains the oligonucleotides used in sequencing. Nucleotide sequence information was compiled using the Gene Jockey II program (Biosoft, Cambridge, UK). Searches of the NCBI database were performed using the BLAST X program (Gish *et al* 1993)

2.2.10 IPCRM

Before IPCRM, 5 µg of the template DNA was denatured by the addition of 10 µl of denaturing solution (1M NaOH, 1mM EDTA) and incubated at 37°C for 15 mins. The solution is neutralised by the addition of 5 µl of 3 M Sodium Acetate [pH 4.8]. The resulting DNA was precipitated with on ice 100% EtoH, washed in 70% EtoH and resuspended in 20 µl dH_2O (Dorrell *et al* 1996). IPCRM cycling reaction was carried out as described in Table 2.3.

Table 2.4 Primers used during this study for PCR

Name	Sequence (5'-3')	Target sequence
9F	GCGCTGCAGTWTTWGTWGTWGAWGA	response regulators
12R	AAAAAGCTTNGKNGGRTTRAANGGYTT	response regulators
13R	GCGAAGCTTTWARRTYAAAWGGTTT	response regulators
15F	GCGCTGCAGTWYTWDTWGTRTAWGAT	response regulators
17R	GCGAAGCTTAARATNCTNTAYGAYGA	response regulators
18R	GCGAAGCTTTWARRTYAAAWGGTTT	response regulators
V1	TGTAAAACGACGGCCAGT	insert in pUC19
V2	ATGTTGTGTGGAATTGTG	insert in pUC19
Kan F	AGCGAACCATTTGAG	kanamycin specific
Kan R	ACTTACTTTGCCATC	kanamycin specific
SPCY1	GCTTCTAATGCTGAGAT	*cheY*1 gene
SPCY2	AACCATCCAATGACCCT	*cheY*1 gene
SPCY3	TAAAAGGAGAAGCGC	*cheY*1 gene
SPCY4	CATTGGCTTTAACACTC	*cheY*1 gene
IPCY1	TCAGATCTGTTCATTTCAGGCAT	*cheY*1 gene
IPCY2	CGAGATCTAGGTGCGCTCCGATAGC	*cheY*1 gene
SPCA1	GCGGTGCAGAGAACCCTGTGATGC	*cheA* gene
SPCA2	GCGAAGCTTTGGATTCGTTGATCA	*cheA* gene
IPCA1	CGAGATCTAACACTGAAGAAAGCGGC	*cheA* gene
IPCA2	CAAGATCTATGTACACCGTTGATGG	*cheA* gene
C3.4	GTGGGCGTTCAAGAAGAG	*cheA* gene
SPF1	TTAGAGGCGACTAAC	*cheY* gene
SPF2	CGATTGGTCTCCTTC	*cheY* gene
IPCF1	TAAGATCTCCTGCCTTTGATCGC	*cheY* gene
IPCF2	GAAGATCTGAATTTATTGTATTT	*cheY* gene

Underlined nucleotides represent *Pst*I (CTCCAG), *Hind*III (AAGCTT) or *Bgl*II (AGATCT) sites. K=G or T; R=A or G; Y=C or T; W=A or T; D=A, G or T; N=A, C, G or T.

2.3 Analysis of Genomic or Plasmid DNA by Southern Hybridisation.

The detection of specific sequences separated by agarose gel electrophoresis was performed using protocols based on those described by Southern (1975). For Southern analysis 5-10 μg of genomic DNA and 100-200 ng of plasmid DNA was digested with the appropriate restriction enzyme and resolved on a 0.8% gel as described in section 2.2.3. For target DNA sequences greater than 10 kb, the gel was washed for 10 mins in depurinating solution (0.2 N HCl). Otherwise the depurinating step was omitted and the gel washed in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 25 mins followed by a 30 mins wash in neutralising solution (1.5 M NaCl, 0.5 M Tris-HCl). DNA fragments were transferred from the agarose gel to a nylon membrane (Hybond N, AmershamPharmacia, Little Chalfont, UK) using the Hybaid vacuum blotting apparatus (Hybaid, Middlesex, UK) for 2 hours and the DNA fixed to the membrane by baking at 80°C for 2 hours.

2.3.1 Preparation of Radiolabelled Probes

Restriction fragments and PCR products used as probes were isolated from low-melting point temperature agarose as described in 2.2.4. Probes were radiolabelled in agarose by primer extension with random nonamer primers using a Rediprime DNA labelling kit (AmershamPharmacia) according to manufacturers instructions.

2.3.2 Hybridisation and Detection

Pre-hybridisation and Hybridisation were carried out at 65°C in Rapid-hyb buffer (AmershamPharmacia) for 30 mins and 2 hrs respectively. After hybridisation the membrane was transferred to a large volume (200 mls) of stringency wash 1 buffer (2X SSC, 0.1% SDS) at room temperature and washed for 20 mins. The membrane was then washed twice in stringency wash 2 buffer (0.5X SSC, 0.1%

SDS) at 65°C for 15 mins. Excess stringency wash 2 was removed, the blot wrapped in cling-film and exposed to Hyperfilm-MP (AmershamPharmacia).

2.4 Screening of λZAPII Express *H. pylori* genomic library.

A genomic library of *H. pylori* NCTC 11637 in the vector λZAPII was a gift from Dr. C. Penn, University of Birmingham. This library was constructed by ligation of *Sau*3AI partial digest fragments of *H. pylori* chromosomal DNA (of average 4.5 kb) to *Bam*HI-digested lambda arms. The library was stored at 4°C in SM buffer (0.1 M NaCl, 0.008 M $MgSO_4$, 0.05 M Tris-HCl [pH 7.5], 2% gelatin) containing 0.02% chloroform.

2.4.1 Plating plaques.

E. coli XL1-Blue MRF' which was used for plating the bacteriophage library was grown overnight in LB broth containing 0.2% Maltose and 10 mM $MgSO_4$ at 30°C. The bacterial cells were harvested by centrifugation and resuspended in 10 mM $MgSO_4$ to an OD_{600} of 0.5 (UltrospecIII, Amersham Pharmacia). 200 µl aliquots of the cells were mixed with a dilution of the bacteriophage stock that resulted in a number of plaques that could be individually visualised on the plate for subsequent screening (~100-150 pfu per 90 mm plate). This was then incubated for 15 mins at 37°C to allow for transfection. The transfected bacterial cells were added to 3 ml of molten (48°C) NZY top agar (0.5% NaCl, 0.2% $MgSO_4.7H_2O$, 5% Yeast extract, 1% NZ amine, 0.7% agarose) and poured onto the plates containing standard agar. Plates were incubated overnight at 37°C to allow plaque formation.

2.4.2 Screening procedure

To prevent the top agar from sticking to the nitrocellulose filter the plates were refrigerated for 2 hrs at 4°C. The plaques were transferred for 2 mins onto

nitrocellulose and their orientation marked using a needle. The filters were denatured 2 mins followed by neutralising for 5 mins as described in section 2.3. Finally the filters were rinsed for 30 secs in (0.2 M Tris-HCl [pH 7.5] 2X SSC), blotted briefly on Whatmann 3 MM paper (Whatmann International, Maidstone, UK) and baked for 2 hrs at 80°C. Probe preparation, hybridisation and detection was carried out as described in sections 2.3.1 and 2.3.2. Plaques which hybridised to the probe were carefully cored and stored at 4°C in SM buffer containing 0.02% chloroform.

2.4.3 Excision of positive pBK-CMV plasmids

E. coli XL1-Blue MRF' was grown in LB broth overnight to an OD_{600} of 0.2-0.5. The culture was centrifuged to rescue the bacterial cells and resuspended in 10 mM $MgSO_4$ to OD_{600} of 1.0. 200 µL of these cells were mixed with 200 µl of phage stock (containing > $1x10^6$ phage particles) and 1 µl of ExAssist helper phage (Stratagene) and incubated at 37°C for 15 mins. 3 ml of LB broth was added and incubated with shaking for 2-2.5 hrs at 37°C during which time the recombinant pBK-CMV phagemids are excised packaged as filamentous particles and secreted from the *E. coli* cells. The mixture was heated to 70°C for 15 mins and spun at 4000 x g for 15 mins to destroy the *E. coli* cells and remove the subsequent cell debris. 10-100 µl of the supernatant which contained the rescued phagmid was then added to 200 µl of freshly grown *E. coli* XLOLR cells (OD_{600} of 1.0) and incubated at 37°C for 20 min. LB broth (300 µl) was added and incubated at 37°C for 45 mins. 200 µl of each sample was plated onto LB-kanamycin (50 µg/ml) plates and incubated overnight at 37°C.

2.5 *H. pylori* Techniques

2.5.1 Chromosomal Preparation of *H. pylori*

Genomic DNA was isolated using a method described by Pitcher *et al* (1989). Three plates of *H. pylori* (2-3 days growth) where resuspended in 500 µl of GES reagent (5 M guanidine thiocyanate, 0.1 M EDTA, 30% Sacrosyl) for 60 mins. Ammonium acetate was added to the final concentration of 2.5 M and the samples were mixed and incubated on ice for 10 mins. To extract proteins, 500 µl of chloroform: isoamyl (24:1) was added, and the mixture was centrifuged for 5 mins at 13,000 rpm. The resulting aqueous-layer was transferred to a fresh eppendorf tube and precipitated with 0.45 volumes of iso-propanoyl at room temperature. The chromosomal DNA was washed twice with 500 µl of 70% EtoH at room temperature. Following drying the pellet was then re suspended in 80-100 µl of distilled H_2O.

2.5.2 Natural Transformation of *H. pylori* cells

H. pylori cells grown for 2-3 days on blood-Dent agar plates were harvested in BHI medium containing 10% FCS and diluted to an OD_{550} of 0.1 (approx. 3 $x10^7$ bacteria ml^{-1}). The cultures were incubated overnight, in micro aerophilic conditions, and the optical density checked to ensure the multiplication of the bacteria. 1-5 µl of super-coiled DNA (1-5 µg total) was added and after a further incubation of 12 hrs, aliquots of 100 µl were spread on blood-Dent agar plates containing the appropriate selective antibiotic (kanamycin 50 µg/ml, chloramphenicol 6 µg/ml). Antibiotic resistant transformants were obtained after 4-5 days incubation under microaerophilic conditions at 37°C.

2.5.3 Electroporation into *H. pylori*

Preparation of Electrocompetent cells for *H. pylori*

H. pylori grown for 2-3 days on blood-Dent agar plates were resuspended in 20 mls of electroporation buffer (272 mM Sucrose, 15% glycerol) followed by centrifugation at 2000 rpm for 5 mins. This was repeated twice and the cells were finally resuspended in 500 µl-1 ml of electroporation buffer (density of 10^{10}-10^{11} bacteria ml^{-1}). These cells can be stored for up to 9 months at -70°C.

Electroporation of *H. pylori* cells

1-10 µl of DNA (1-5 µg total) was added to a pre-chilled cuvette containing 50 µl of defrosted *H. pylori* electrocompetent cells and chilled on ice for 5-10 mins. The cuvette was dried and pulsed in the electroporation apparatus (Biorad, UK) at 200 ohms, 25 µFD and 2.5 $kVcm^{-1}$. The electrophorated cells were immediately resuspended in 100 µl of SOC medium (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM $MgSO_4$ and 20 mM glucose) and plated out onto blood-Dent plates without antibiotic selection at 37°C under microaerophilic conditions. Once growth was produced on non-selective plates, cells were resuspended in BHI and plated onto selective antibiotic plates as defined in section 2.3.2. Antibiotic resistant transformants were obtained after 4-5 days incubation under microaerophilic conditions at 37°C.

2.6 *In vitro* and *in vivo* analysis of *H. pylori* chemotaxis mutants

2.6.1 Swarm plates

Bacterial motility was assayed on 0.3% agar plates containing BHI broth supplemented with 10 % FCS. Plates were seeded with 10 µl of overnight broth culture and the plates incubated for 2-3 days at 37°C. Results were recorded on the basis of swarm diameter.

2.6.2 Computerised Video Tracking

Computerised video tracking was used to analyse free-swimming cells. Motile cells grown in culture to OD_{600} 0.4 were drawn into 100 μm diameter optically flat micro slides (Camlab Ltd, Cambridge, UK) and one end of the slides was sealed with vinyl plastic putty (Critoseal, Hawsksley, UK) to prevent bacterial cells from drifting. Slides were observed with a Zeiss Standard 14 phase contrast microscope at 37°C. Once it was confirmed that the cells were motile, 200 μl volumes of cells were briefly spun (4000 rpm for 30 secs) and then resuspended in an equal volume of potassium phosphate (10 mM [pH 7]). These cells were drawn into micro slides and there motility checked. Free swimming tracks were determined by motion analysis using the Hobson BacTracker system (Hobson Tracking Systems Ltd, UK.). Prior to analysis limits were set using dead bacteria to prevent brownian motion been interpreted as motile bacteria. This system provides detailed analysis of various parameters to describe motility of the bacteria, including the curvilinear speed (CVS, the speed of the bacterium along its path) and the straight line velocity (SLV, the speed of the bacterium in a straight line from the beginning to the end of its path). Bacterial cells were also monitored after the addition of urea. 5 μl of urea (10X concentration) was added to 45 μl of resuspended bacterial cells and mixed, these cells were immediately analysed as before. Individual free swimming cells were monitored for ~ 2 s, and the mean values of 100-200 cells were determined for both samples of at least six replicates. Statistical significance was determined by the independent t-test.

2.6.3 Capillary Assay (Modified Adlers chemotaxis system)

This method was adapted from that originally designed by Adler (1973). Bacterial strains were grown overnight to log phase, the OD_{600} measured and their motility checked. The bacterial cells were harvested by centrifugation (1min @ 13,000rpm) and resuspended in chemotaxis buffer (0.2 M Na_2HPO_4, 0.1 M citric

acid) to 10^6 bacterium/ml (~ OD_{600} 0.1). The tips of capillary tubes (50 µl vol; Sigma, Poole, UK) were then filled with hog gastric mucin (Sigma, Poole, UK) or chemotaxis buffer (control), sealed at one end and inserted vertically into 0.5 ml eppendourf tubes containing 300 µl of resuspended bacterial cells. These were incubated horizontally under microaerphilic conditions for 45 mins at 37°C. After incubation, the tubes were disassembled and the lower 10 mm of liquid content discarded. The number of bacteria remaining in each capillary tube were then determined by serial dilution, culture and counting the number of resultant colonies. All assays were performed in triplicate on at least three separate occasions. The results were expressed as the chemotaxis ratio R_{che} (CFUs/ml in taxin capillary : CFUs/ml in control capillary) to normalise experimental data (Moulton and Montie 1979). Soluble hog gastric mucin (Sigma) was prepared as 1 %, 0.5 % and 0.1 % solution in chemotaxis buffer.

2.6.4 SDS PAGE gel analysis

2.6.4.1 1D SDS PAGE gels

Preparation of proteins

Bacterial strains were grown overnight to log phase and the OD_{600} measured. The bacterial cells were harvested by centrifugation washed in dH_20 and resuspended in 50 µl of 1X SDS PAGE loading buffer (50 mM Tris-HCL [pH 6.7], 2% 2-mercaptoethanol, 2% SDS, 0.08% (w/v) bromophenol blue, 40% (v/v) glycerol). Protein samples containing 40 µg, measured by BCA™ Protein Assay Reagent (Pierce, Illinois, USA) were boiled for 5 mins prior to loading on the 1D SDS PAGE gel.

Pouring 1D SDS PAGE gel

The 12% resolving gel acrylamide solution was prepared as described in Table 2.5 and poured immediately into the gel setting apparatus. The resolving gel was

overlaid with iosbutanol and left to polymerise for 60 mins. The isobutanol was removed and the 12% stacking gel prepared as described in Table 2.5 and immediately poured onto the surface of the resolving gel. A comb was inserted into the stacking gel and the gel allowed to set for 60 mins. Equal concentrations of proteins were loaded into each lane. A constant current of 50 mA was applied to the SDS PAGE gel for 4 hrs or until the dye front had reached the bottom of the gel.

Table 2.5 Preparation of a resolving and stacking gel for a 1D SDS PAGE gel

Reagent	Resolving gel	Stacking gel
Protogel	20 ml	1.3 ml
Protogel buffer	13 ml	
10% Ammonium persulfate	1 ml	0.05 ml
Temed	0.1 ml	0.01 ml
Protogel Stacking buffer		2.5 ml
dH_2O	16.5 ml	6.1 ml
Total volume	50 ml	10 ml

Once electrophoresis of the SDS-PAGE gel was complete the gel was removed from the electrophoresis apparatus and transferred into a solution of coomassie brilliant blue (0.25 g of coomassie brilliant blue in 90 ml methanol:H_2O (1:1 v/v) and 10 ml of glacial acetic acid) and stained for a minimum of 4 hrs. The coomassie blue stain was removed and the gel washed in destain solution (12% (v/v) methanol, 10% (v/v) glacial acetic acid) overnight.

2.6.4.2 2D SDS PAGE gels

Preparation of protein sample and Immobiline dry strip gel rehydration

The volume of bacterial culture (OD_{600} of 1.0) required to give 30 µg of protein was pelleted and resuspended in an equal volume of water and Lysis buffer (urea 13.5g, Triton X-100 0.5 ml, 2-mercaptoethanol, IPG buffer 25 µl (AmershamPharmacia), phenylmethanesulfonyl fluoride 35 mg and dH_2O to 25 ml) and incubated at room temperature for 30 mins. The protein sample was harvested by centrifugation (50,000 rpm for 15 mins) and the supernatant carefully removed avoiding the pellet. For each 18 cm Immobiline dry strip (pH 3-10 NL) (AmershamPharmacia) 360 µl of rehydration solution (urea 2.4 g, TritonX 100 µl, dithiothreitol 75 mg, bromophenol blue, IPG buffer 25 µl and dH_2O to 5 ml) was added to the protein sample and the mixture carefully added to the reswell tray lane, avoiding bubbles. The Immobiline dry strip was carefully placed on top of the protein sample in the reswell tray, covered with mineral oil to prevent evaporation and urea crystallisation, and allowed to hydrate overnight at room temperature.

First Dimension run

The rehydrated gel strips were transferred to the Immobiline strip aligner which has been placed on the Multiphor II unit according to manufacturers instructions (AmershamPharmacia). Up to 12 strips can be run in the first dimension at any one time. Two IEF Electrode strips, soaked with dH_2O, placed on top of the aligned strips at the cathode and anode. The electrodes are pressed down on top of the IEF Electrode strips and 70-80 ml of mineral oil is poured into the tray to completely cover the strips. The gel was ran according to manufacturers instructions (AmershamPharmacia).

After the first dimension, the strips can be stored at -80 °C for later use. The strip to be run in the second dimension was equilibrated in two different Equilibration solutions (ES) (Tris-HCL [pH 6.8] 20 ml, urea 72 g, glycerol 60 ml, 20 ml of 10% SDS, dH_2O to 200 ml) which contained in ES1 (dithiothreitol 25-200 mg/10 ml)

and ES2 (0.45 g Iodoacetamide/10 ml (Sigma), few grains of bromophenol blue/ 10 ml). Each step was carried out for 10 mins. The strip was then blotted and placed on the assembled Excel Gel (12-14%) and the second dimension was performed according to manufacturers instructions (AmershamPharmacia).

The gel was carefully removed from the Multiphor II unit and silver staining was performed according to the manufacturers instructions (AmershamPharmacia). The gel was covered with a cellophane preserving sheet and allowed to dry gel side up on a glass plate at room temperature.

2.6.5 Animal studies

2.6.5.1 Colonisation of gnotobiotic piglet model

Gnotobiotic piglet experiments were carried out essentially as described by Karkwoka *et al* (1987). Large white hybrid piglets (Royal Veterinary College) were derived by caesarean section performed in a sterilised isolator unit. The piglets were maintained in sterile isolator units and rectal swabs were cultured from the piglets to demonstrate sterility before inoculation. To suppress secretion of stomach acid, piglets were given 40 mg cimetidine (Tagamet; SmithKline Beecham, UK.), orally, 1 hour before inoculation of bacterial suspensions. This was repeated 6 hours after inoculation. Animals were challenged orally at 2 days of age with 2 mls of 10^9 cfu bacteria grown in individual broth cultures for 12 h. After seven days the piglets were sacrificed. The mucosa from half of the stomach was removed, weighed and homogenised and the extent of the bacterial colonisation quantified by serial plate dilution on Dent agar plates (see section 2.1).

2.6.5.2 Colonisation of mouse model

Female outbred mice (HSD/ICR strain, Harlan Ltd, Bicester, UK.) of approximately 20 g body weight (4-6 weeks old) were challanged orally on

successive days. Prior to challange, all strains were pretreated with acidfied 5 mM urea (pH 2) in order to boost urease activity and thus optimise colonisation potential (McColm 1997). Challange inocula were 1 ml volumes of 24 hour Tryptose Soya broth cultures containing between $1x10^7$ and $1x10^8$ colony forming units. At 2 and 8 weeks, 10 mice from each group were culled by CO_2 inhalation, then the stomachs removed and opened along the greater curvature. After briefly washing to remove the stomach contents, the entire mucosal surface was spread evenly over the surface of a Columbia chocolate agar plate containing selective antibiotics (amphotericin B (50 μg/ml), vancomycin (100 μg/ml), polymyxin B (3.3 μg/ml), bactracin (200 μg/ml) and nalidixic acid (10.7 μg/ml)) and incubated microaerobically for 7 days at 37°C. The culture plates were then evaluated for growth using the scoring system outlined below in Table 2.6 (McColm 1997).

Table 2.6 Scoring system for Visual Assesment of Cultures. For a group of 10 mice the maximum score is 50 (McColm 1997).

No. of Colonies present	Score
0	0
1-20	1
>20-<50	2
>50-<100	3
>100-<200	4
>200	5

Chapter 3

Amplification, cloning, sequencing and analysis of the *cheY*1, *cheA* and *cheY* chemotaxis components from *H. pylori*

3.1 Introduction

Chemotaxis, the purposeful movement of bacteria to and from chemical stimuli, has been most extensively studied in *E. coli* and *S. typhimurium* (Stock and Surette 1996, Falke *et al* 1997). In these organisms, the two component regulatory system, CheA and CheY, has been shown to play a central role in chemotactic behaviour. Autophosphorylation activity of the histidine protein kinase is controlled by methyl accepting chemotaxis proteins receptors (MCPs). A coupling protein, CheW, forms a ternary complex with CheA and the MCP cytoplasmic domain, allowing phosphorylation to occur. CheA in turn regulates the phosphorylation and dephosphorylation of response regulator CheY, which interacts with the flagellar motor switch complex to control swimming behaviour (Eisenbach, 1996). The mechanism of chemotaxis in different bacteria is generally based on the same basic principle, although as outlined in Chapter 1, the details of the mechanism, function and numbers of the various components present, do vary between bacterial species.

Chemotaxis is a vital adaptation for many bacteria capable of colonising mucosal surfaces. A chemotactic response to urea, bicarbonate and mucin by *H. pylori* has been demonstrated (Mizote *et al* 1997, Turner *et al* 1997). However very little is known about the mechanism of the chemotactic response or of its role played in the motility and virulence of *H. pylori*. The initial aim of this project was to amplify, clone and sequence putative *cheY* and *cheA* homologues from *H. pylori* NCTC 11637.

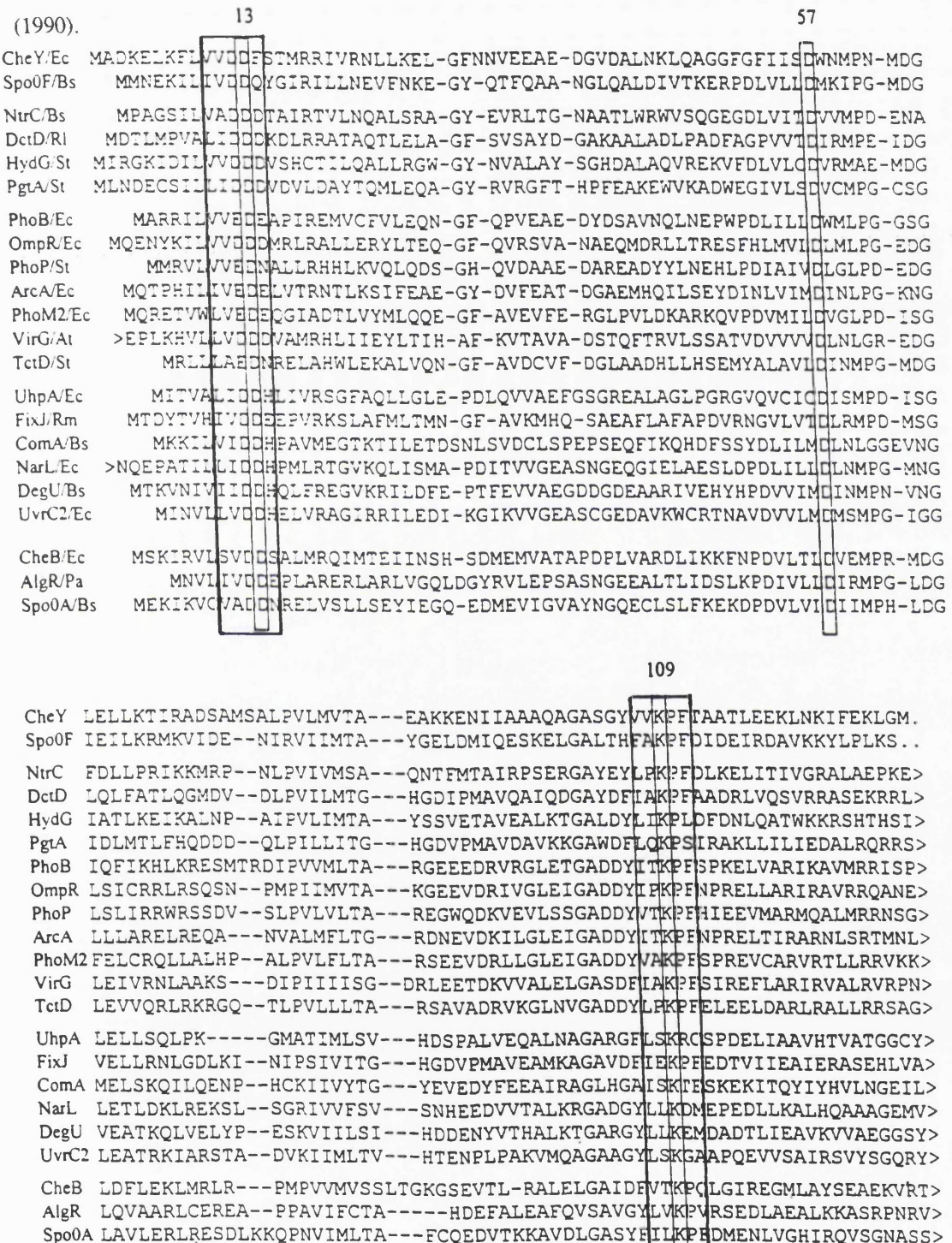
3.2 Results

3.2.1 Amplification and sequencing of *cheY*1, *cheA* and *cheY*

3.2.1.1 Design of degenerate primers to amplify response regulators by PCRDOP.

Degenerate primers were designed based upon conserved amino acids or regions of patch homology within N-terminal regions of known response regulators (Fig 3.1). All degenerate primers used in this study were designed to amplify gene fragments encoding a 330 bp portion of the N-terminal region of a response regulator from *H. pylori*. To expedite cloning of amplified gene fragments, a *Pst*I (CTGCAG) restriction site was incorporated at the 5' terminus of the forward degenerate primer and a *Hind*III (AAGCTT) restriction site at the 5' terminus of the reverse degenerate primer. Typically a 3 bp clamp was included in the design of the degenerate primer to ensure optimal digestion at the restriction site. The 3' region of the primers were designed from amino acid residues conserved throughout all members of the response regulator family, but primarily using those systems which have been demonstrated to be involved in the virulence of bacteria. For example, degenerate primers 9F, 15F, 12R and 18R were designed from conserved amino acid motifs found in predominantly OmpR and PhoP systems of *E. coli* and *S. typhimurium* (Stock *et al* 1990). The remaining primers, 13R and 17R, were designed using conserved regions found in the NtrC system of *B. subtilis* and the HydG system of *S. typhimurium*, respectively (Stock *et al* 1990). All degenerate primers, with the exception of 12R, were designed to reflect the predicted codon bias, 69% A+T, of the *Helicobacter* species. The design features of the primers utilised during this study are summarised in Table 3.1.

Figure 3.1: Multiple alignment of the N-terminal domains of some of the members of the response regulator super-family. Individual response regulator proteins are grouped according to subfamily. Boxed regions indicate the location of the three conserved residues. Response regulator sequences are from *E. coli* (Ec), *B. subtilis* (Bs), *R. leguminosarum* (Rl), *S. typhimurium* (St), *A. tumefaciens* (At), *R. meliloti* (Rm) and *P. aeruginosa* (Ps) as indicated. Adapted from Stock *et al* (1990).



3.2.1.2 Amplification of response regulators by PCRDOP

A combination of degenerate primers (Table 3.1) were used during PCRDOP experiments as described in section 2.2.8. To reveal the optimum conditions for amplification, PCRDOP reactions were carried out with annealing temperatures ranging from 30°C to 45°C at 5°C intervals. Amplified products were resolved by agarose gel electrophoresis as described in section 2.2.5. At low annealing temperatures considerable 'smearing' was observed, probably due to mispriming with the *H. pylori* chromosomal DNA. The degeneracy of the primers and the low annealing temperatures failed in the majority of reactions to produce a definitive PCR product. However, because the predicted size of the product was known, the appropriate sized bands could be excised and gel purified as described in section 2.2.6. Numerous products were amplified using the combination of primers listed in Table 3.1. A 330 bp product amplified using degenerate primers 15F/12R (Fig. 3.2) was gel isolated, cloned and sequenced. Sequence analysis revealed that a fragment of a gene homologous to a response regulator had been amplified. The deduced amino acid sequence had the highest homology (47% identity to *E. coli* CheY) to the CheY homologue from a range of bacteria. The *H. pylori* gene fragment was designated RR1 and the construct containing this gene fragment was named pCY.

Table 3.1 Summary of the design features of degenerate primers used for PCRDOP experiments.

Primer	Region	RR family	Codon Bias
9F	VVED	PhoP/S.t	A-T
12R	KPFN	OmpR/E.c	Any
13R	KID/NFPK	NtrC/B.s	A-T
15F	VLLVDD	PhoP/S.t	A-T
17R	KILYD/KD	HydG/S.t	A-T
18R	EIDFPK	PhoP/S.t	A-T

Figure 3.2

PCRDOP experiments on *H. pylori* NCTC11637 DNA at 40°C annealing temperature. Amplified products were analysed by electrophoresis on a 1% agarose gel. Lane 1, 1 kb molecular mass ladder, lane 2, 9F/12R, lane 3, 15F/12R, lane 4, 9F/13R, lane 5, 15F/17R, lane 6, 15F/18R, lane 7, 9F/17R and lane 8, 9F/18R. Negative controls consisting of PCR mixture and primer combinations (not shown here) were performed for all PCRDOP experiments described in this study. The arrow indicates the band isolated for further study.

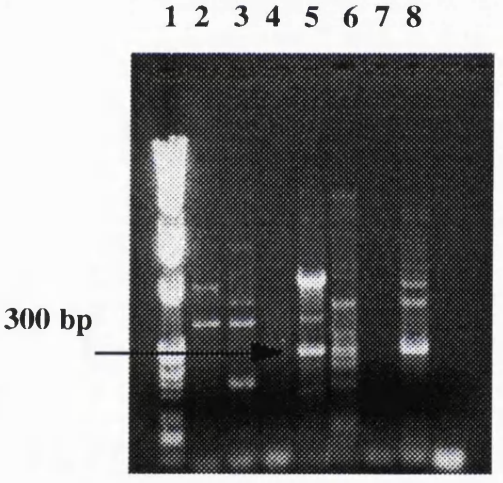


Figure 3.3

Nucleotide and deduced amino acid sequence of *cheY*1 gene fragment. The regions incorporating the degenerate primers are underlined.

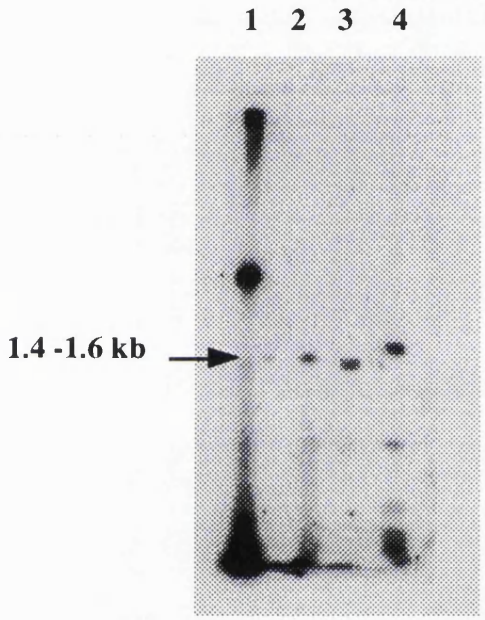
```
TTG GTT GTA GAT GAT AGC TCA ACT ATG AGG AGA ATT ATT AAA AAC ACA CTT TCA CGC
 L   V   V   D   D   S   S   T   M   R   R   I   I   K   N   T   L   S   R

TTA GGC TAT GAA GAT GTT TTA GAA GCT GAG CAT GGG GTG GAA GCT TGG GAG AAA CTA GAC
L   G   Y   E   D   V   L   E   A   E   H   G   V   E   A   W   E   K   L   D

GCT AAT GCG GAC ACT AAG GTG CTT ATC ACA GAT TGG AAC ATG CCT GAA ATG AAC GGC TTA
A   N   A   D   T   K   V   L   I   T   D   W   N   M   P   E   M   N   G   L

GAT CTC GTT AAA AAG GTG CGC TCC GAT AGC CGC TTT AAA GAA ATC CCT ATT ATT ATG ATC
D   L   V   K   K   V   R   S   D   S   R   F   K   E   I   P   I   I   M   I

ACC ACA GAG GGC GGT AAG GCT GAG GTC ATT ACG GCT TTG AAA GCG GGC GTG AAC AAC TAC
T   T   E   G   G   K   A   E   V   I   T   A   L   K   A   G   V   N   N   Y

ATT GTG AAA CCT TTT
I   V   K   P   F
```

3.2.1.3 Confirmation of the origin of the response regulator isolated by PCRDOP, and its conservation in other *H. pylori* strains.

Due to the non-specific nature of PCRDOP it was necessary to confirm that the RR1 gene fragment identified in this study was amplified from *H. pylori* and not from a contaminating exogenous sources of DNA. Southern hybridisation using the response regulator 330 bp gene fragment as a specific probe was carried out as described in section 2.3. The probe hybridised to a 1.6 kb *Hae*III fragment from digested NCTC11637 chromosomal DNA. To determine whether other *H. pylori* strains possess RR1 or homologous sequences, *Hae*III digested genomic DNA from *H. pylori* N6 and 26695 was also screened, using the same probe under conditions of high stringency. The probe hybridised to a single fragment (1.4-1.6 kb) in both the N6 and 26695 strains indicating that the gene is present in different *H. pylori* species (Fig. 3.4)

Figure 3.4

Southern hybridisation of *Hae*III digested chromosomal DNA probed with the 330 bp *Hind*III/*Pst*I fragment of pCY. Lane 1, *Hind*III/*Pst*I digested pCY as a positive control, lane 2, N6, lane 3, 26695 and lane 4, NCTC11637.



3.2.1.4 Screening of a *H. pylori* gene library

In order to isolate the full response regulator gene, a λZAP II Express genomic library of *H. pylori* NCTC 11637 was screened using the insert of pCY as a DNA probe as described in section 2.4. $1.5x10^3$ plaques were screened (equivalent to 2.5 genomes) and 54 clones hybridised strongly to the gene fragment. *E. coli* sub-clones containing the recombinant pBK-CMV plasmids were generated for 10 clones which exhibited strong hybridisation.

3.2.1.5 Analysis of recombinant plasmids by restriction analysis and Southern hybridisation

Ten putative CheY1 recombinant pBK-CMV plasmids were digested with *SpeI*/*EcoR*I (two restriction sites that flank the insert in the plasmid pBK-CMV). The linearised 4.5 kb vector, pBK-CMV, is visible in all lanes. The insert sizes ranged from 2.0-5.8 kb (Fig. 3.5a). To identify a positive *cheY*1 containing clone, the digested products were analysed by Southern blot hybridisation using the 330 bp *Hind*III/*Pst*I restriction fragment of pCY (RR1 fragment cloned into pUC19) as a probe (Fig. 3.5b). Hybridisation was observed to digested clones 18B and 18C (Fig. 3.5b, lanes 8, 9), which contained fragments of 5.2 kb and 3 kb respectively, and to lane 12 which contained the positive control.

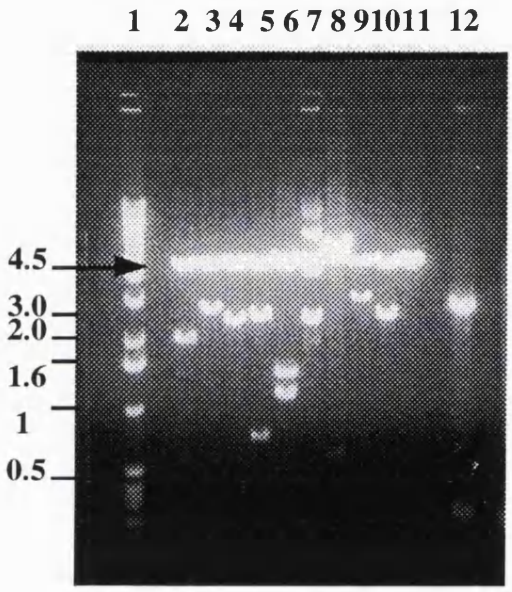
Figure 3.5

(a) 0.8% Agarose gel analysis of *SpeI*/*Eco*RI digest of putative CheY1 recombinant pBK-CMV clones. Lane 1, 1 kb molecular weight marker, lane 2, 13D (insert size 2 kb), lane 3, 16A (insert size 2.8 kb), lane 4, 15C (insert size 2.6 kb), lane 5, 15D (insert sizes 0.7 and 2.7 kb), lane 6, 16B (insert sizes 1.2 and 1.5 kb), lane 7, 18A (partial digest), lane 8, 18B (insert sizes 0.6 and 5.2 kb), lane 9, 18C (insert size 3 kb), lane 10, 18E (insert size 2.7 kb), lane 11, 18F (insert size 4.7 kb) and lane 12, positive control of 330 bp gene fragment.

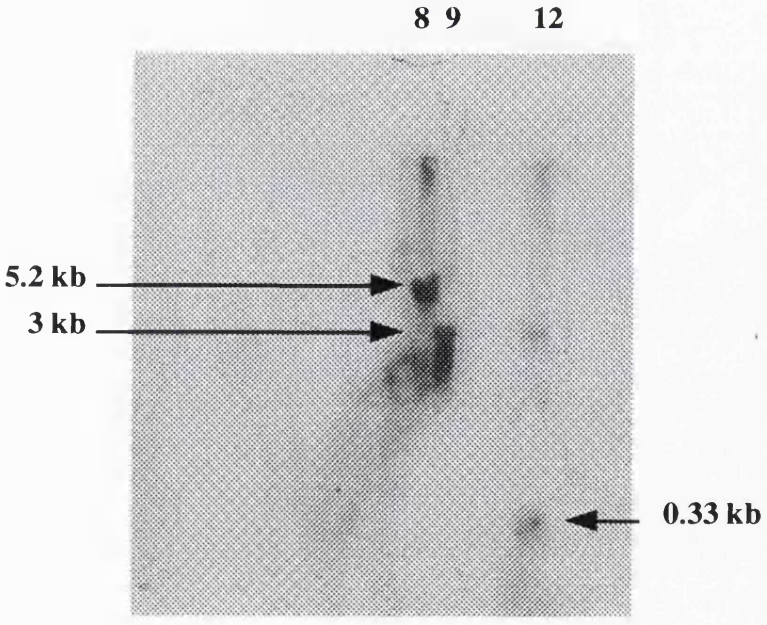
(b) Identification of a recombinant clone containing *cheY*1 sequences, isolated from a λZAP 11637 library, by Southern hybridisation using the RR1 gene fragment as a probe. Lanes as in Fig 3.5 a.

Figure 3.5

(a)



(b)



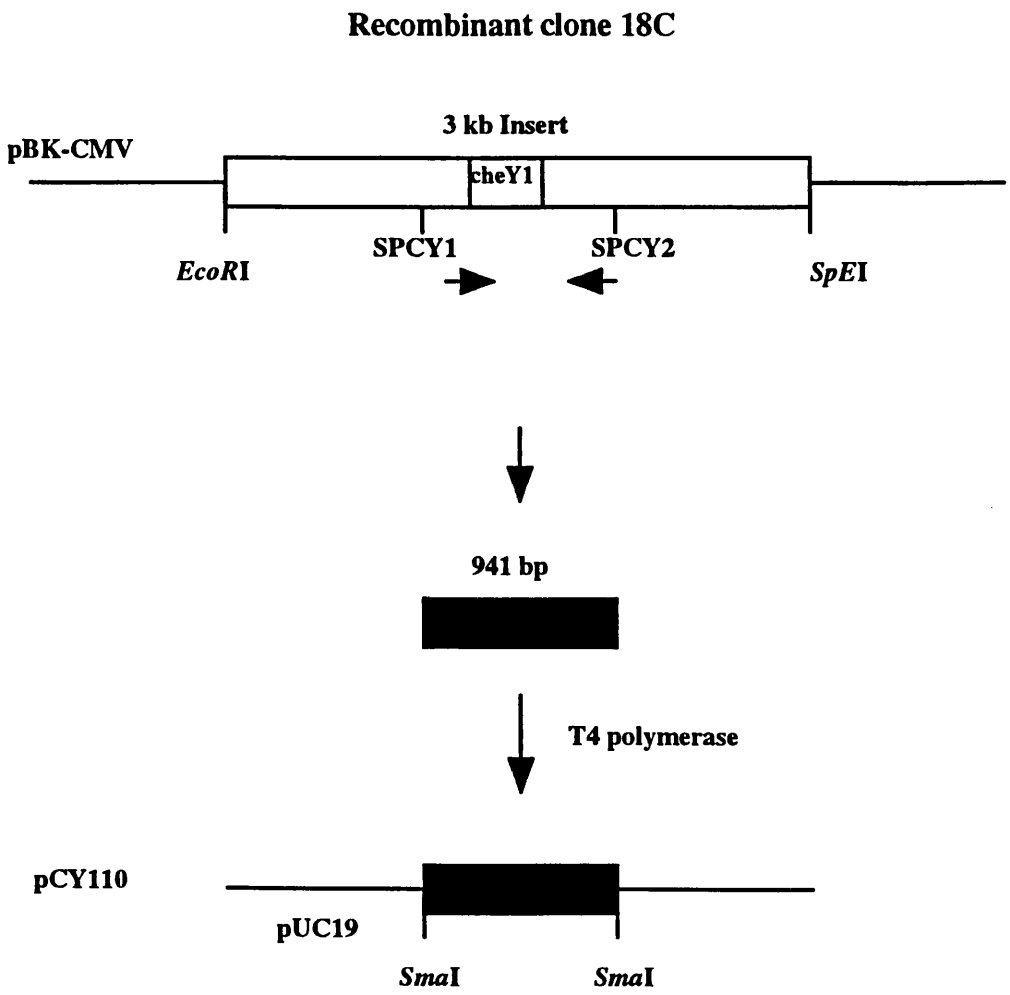


Figure 3.5c: Cloning of *cheY*1. Strategy outlined in section 3.2.1.6

3.2.1.6 Cloning and analysis of the nucleotide sequence of *cheY1*

Recombinant clone, 18C, which contained a 3 kb insert was partially sequenced internally using primers SPCY3 and SPCY4.. Specific primers, SPCY1 and SPCY2, were designed from this sequence information to amplify a 941 bp fragment containing the entire putative *cheY1* gene plus some flanking DNA. The amplified product was treated with T4 polymerase, as described in section 2.2.3, to generate blunt ends. This was cloned into *Sma*I digested pUC19 and designated pCY110. Searches using BLAST X (Gish *et al* 1993) software revealed that the plasmid pCY110 insert had significant identity to CheY from several bacteria. The complete ORF encodes a protein of 124 amino acids with a predicted molecular weight of 13.8 kDa. A possible TTG start codon of the *cheY1* was found at position 132 of the nucleotide sequence (Fig. 3.6). Seven bases upstream from the TTG start codon is a possible ribosomal binding site, (5'-AGGAGA-3'). Other features, also identified by eye, include the -10 and -35 regions and a transcriptional start point (Fig. 3.6)

Figure 3.6

Nucleotide and deduced amino acid sequences of the complete response regulator, *cheY*1. The putative ribosome binding site (boldface), the putative -10 and -35 promoter elements (boldface and underlined) and the corresponding potential transcriptional start point (underlined) are indicated. The possible TTG start and stop codon are indicated by asterisks.

```
1/1                    -35              31/11      -10
ATG ATT ATT GTC GGT CAA GGT ATT TTA TCA AAA CAT GCT ATT ATT TGG ATG ATT TAT TAT
M   I   I   V   G   Q   G   I   L   S   K   H   A   I   I   W   M   I   Y   Y
61/21                                  91/31
TAT AAG GCG TTA GAG ACG CTT GGC TGA AGA GCC TAA TTT AAT GCA ATC TTA ATA AGG AGA
Y   K   A   L   E   T   L   G   *   R   A   *   F   N   A   I   L   I   R   R
121/41  *                              151/51
AGC GCT TTG AAA TTA AAG TTG TTG GTT GTA GAT GAT AGC TCA ACT ATG AGG AGA ATT ATT
S   A   L   K   L   K   L   L   V   V   D   D   S   S   T   M   R   R   I   I
181/61                                 211/71
AAA AAC ACA CTT TCA CGC TTA GGC TAT GAA GAT GTT TTA GAA GCT GAG CAT GGG GTG GAA
K   N   T   L   S   R   L   G   Y   E   D   V   L   E   A   E   H   G   V   E
241/81                                 271/91
GCT TGG GAG AAA CTA GAC GCT AAT GCG GAC ACT AAG GTG CTT ATC ACA GAT TGG AAC ATG
A   W   E   K   L   D   A   N   A   D   T   K   V   L   I   T   D   W   N   M
301/101                                331/111
CCT GAA ATG AAC GGC TTA GAT CTC GTT AAA AAG GTG CGC TCC GAT AGC CGC TTT AAA GAA
P   E   M   N   G   L   D   L   V   K   K   V   R   S   D   S   R   F   K   E
361/121                                391/131
ATC CCT ATT ATT ATG ATC ACC ACA GAG GGC GGT AAG GCT GAG GTC ATT ACG GCT TTG AAA
I   P   I   I   M   I   T   T   E   G   G   K   A   E   V   I   T   A   L   K
421/141                                451/151            *
GCG GGC GTG AAC AAC TAC ATT GTG AAA CCT TTT ACC CCC CAA GTT TTG AAA GAA AAA TTA
A   G   V   N   N   Y   I   V   K   P   F   T   P   Q   V   L   K   E   K   L
481/161
GAG GTT GTT TTA GGG GCA AAC GAT TGA
E   V   V   L   G   A   N   D   *
```

3.2.1.7 Analysis of the deduced amino acid sequence of *cheY*1.

The highest amino acid identity was found to the *C. jejuni* CheY(81%), this was followed by the *P. aeruginosa* CheY homologue (56%). The DNA sequences and protein translations of CheY homologues from several bacterial species have identified three highly conserved aspartate residues, Asp 12, Asp 13 and Asp 57 (the site of phosphorylation) and a single conserved lysine residue, Lys-109 (*E. coli* CheY numbering) (Volz 1993). The three-dimensional structure of CheY from both *E. coli* (Volz and Matsumara 1991) and *S. typhimurium* species (Stock *et al* 1989) has been resolved. Importantly all the sites of structural importance are conserved in the *H. pylori* CheY1 at Asp 7, Asp 8, Asp 53 and Lys 112. Amino acids 90-114 are also highly conserved. It is proposed that this domain is the area

were interaction between the phosphorylated CheY and the FliM or "switch" protein occurs (see section 1.7.3) (Volz 1993) (Fig.3.7).

Figure 3.7 Multiple alignment of CheYs. Sites found conserved, Asp 12, Asp 13, Asp 57 and Lys 109, are all indicated by bold lettering. Sequences were aligned using Clustal V multiple alignment software. Identical amino acids (dots) and conserved changes (vertical lines) are indicated.

```
                        10        20        30        40        50        60
                        |         |         |         |         |         |
                   |  ·|·|··· |·····||· · ·· | ··||· | · · | |||·····
H.pylori 11637     L-----KLLVVDDSSTMRRIIKNTLSRLGYEDVLEAEHGVEAWEKLDANADTKVLITDWNMPE
C.jejuni           M-----KLLVVDDSSTMRRIIKNTLTRLGHDDVLEAEHGVEAWDLLTKNEDVKVLITDWNMPE
P.aeruginosa       M-----KILIVDDFSTMRRIIKNLLRDLGFTNTAEADDGTTALPMLHS-GNFDFLVTDWNMPG
P.putida           M-----KILIVDDFSTMRRIIKNLLRDLGFTNTEEADDGTTALPMLEN-GHYDFLVTDWNMPG
E.coli             MADKELKFLVVDDFSTMRRIVRNLLKELGFNNVEEAEDGVDALNKLQA-GGYGFVISDWNMPN
S.thypimurium      MADKELKFLVVDDFSTMRRIVRNLLKELGFNNVEEAEDGVDALNKLQA-GGFGFIISDWNMPN
B.pseudomallei     M-DKSMKILVVDDFPTMRRIVRNLLKELGYSNVDEAEDGLAGLARLRG-GGYDFVISDWNMPN

                        70        80        90        100       110       120
                        |         |         |         |         |         |
                   | ·| ||| |·|      |·||·|·|·| | ||· · |·· | ·|·····|  · ··|| ||
H.pylori 11637     MNGLDLVKKVRSDSRFKEIPIIMITTEGGKAEVITALKAGVNNYIVKPFTPQVLKEKLEVVLG
C.jejuni           MNGLELVKKVRAEKKYEDMPIIMVTTEGGKAEVITALKAGVNNYIVKPFTPQVLKEKLEDVLG
P.aeruginosa       MTGIDLLRAVRADERLKHLPVLMVTAEAKRDQIIEAAQAGVNGYVVKPFTAQVLKEKIEKIFE
P.putida           MSGIDLLRKVRASEKLKTMPVLMVTAEAKRDQIIEAAQAGVNGYVVKPFTAQVLKEKIEKIFE
E.coli             MDGLELLKTIRADGAMSALPVLMVTAEAKKENIIAAAQAGASGYVVKPFTPATLEEKLNKIFE
S.thypimurium      MDGLELLKTIRADSAMSALPVLMVTAEAKKENIIAAAQAGASGYVVKPFTAATLEEKLNKIFE
B.pseudomallei     LDGLAMLKEIRADASLTHLPVLMVTAESKKENIIAAAQAGASGYVVKPFTAATLDEKLNKILE
```

3.2.1.8 Cloning and analysis of the *cheA* gene fragment.

Random sequencing of a *H. pylori* NCTC 11637 gene library had identified a clone, pILLCA, which contained a putative *cheA* homologue (A. Labigne personal communication). Clone pILLCA (a gift from A. Labigne) was partially sequenced by chromosome walking and protein data base searches revealed extensive homology to *E. coli* CheA. Specific primers, SPCA1 and SPCA2, where designed to amplify a 707 bp fragment of *cheA* from *H. pylori* chromosomal DNA, which was cloned into pUC19 and termed pCA110. The availability of the total genome sequence data of *H. pylori* 26695 enabled confirmation that the gene fragment cloned encoded a portion of the CheAY from *H. pylori*. Comparison of

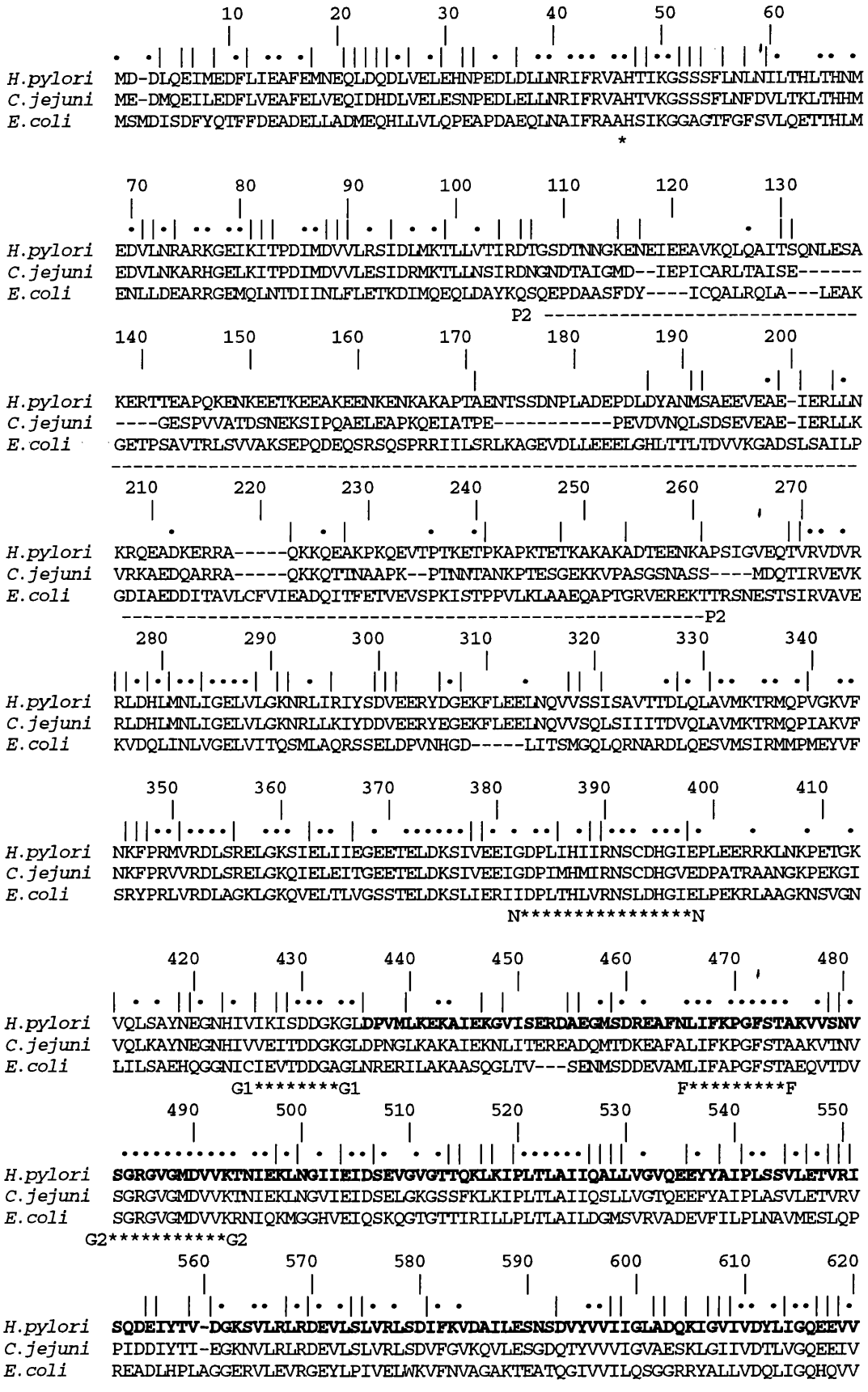
the deduced amino acid sequence of the cloned *cheA* gene fragment from *H. pylori* NCTC11637 to the complete CheA from *H. pylori* 26695 (HP0392) (Tomb *et al* 1997) revealed 97% identity.

3.2.1.9 Analysis of the deduced amino acid sequence of *H. pylori* 26695 CheA.

The highest percentage identity of the *H. pylori* 26695 CheA was to the *P. putida* CheA homologue (46.5%). A 41% identity was found to the *E. coli* CheA. This included the active domains of CheA, which were totally conserved between the *E. coli* and *H. pylori* 26695 proteins (Fig.3.8). The area adjacent to His 48, the site of autophosohorylation, is relatively conserved. Other areas conserved include four blocks of residues involved in kinase function, N (aa 368-384), G1 (aa 422-430), F (aa 461-471), G2 (aa 477-488), found at the carboxyl end, which indicate that this protein is functional as a transmitter. The main area of divergence between the *H. pylori* CheA and the *E. coli* CheA, was shown to be between amino acids 109-260 (*E. coli* numbering). This region contains the P2 domain (CheA 124-257) which has CheY binding capabilities (Stock and Surette 1996). The genome of *Campylobacter jejuni* (NCTC11168) is currently been sequenced (http://www.medmicro.mds.qmw.ac.uk/campylobacter/). The CheA domains of CheAY from both *H. pylori* and *C. jejuni* have a high level of identity (65%) (Fig. 3.8).

Figure 3.8

Alignment of CheA protein sequences from *H. pylori* 26695 (HP0392), *C. jejuni* and *E. coli*. Identical residues are indicated by dots. Features conserved in all three proteins, the regions involved in kinase function (N, G1, F and G2 boxes) and the sites of phosphorylation (H box) are indicated by asteriks. The main differences, from 109-260 (P2 region), are highlighted by dashed lines, and the original fragment of CheA isolated and cloned from *H. pylori* 11637, aa 309-584 (*H. pylori* numbering) is shown in bold print. Sequences were aligned using Clustal V multiple alignment software.


H.pylori MD-DLQEIMEDFLIEAFEMNEQLDQDLVELEHNPEDLDLLNRIFRVAHTIKGSSSFLNLNILTHLTHNM
C.jejuni ME-DMQEILEDFLVEAFELVEQIDHDLVELESNPEDLELLNRIFRVAHTVKGSSSFLNFDVLTKLTHHM
E.coli MSMDISDFYQTFFDEADELLADMEQHLLVLQPEAPDAEQLNAIFRAAHSIKGGAGTFGFSVLQETTHLM
H.pylori EDVLNRARKGEIKITPDIMDVVLRSIDLMKTLLVTIRDTGSDTNNGKENEIEEAVKQLQAITSQNLESA
C.jejuni EDVLNKARHGELKITPDIMDVVLESIDRMKTLLNSIRDNGNDTAIGMD--IEPICARLTAISE------
E.coli ENLLDEARRGEMQLNTDIINLFLETKDIMQEQLDAYKQSQEPDAASFDY----ICQALRQLA---LEAK
P2 ----------
H.pylori KERTTEAPQKENKEETKEEAKEENKENKAKAPTAENTSSDNPLADEPDLDYANMSAEEVEAE-IERLLN
C.jejuni ----GESPVVATDSNEKSIPQAELEAPKQEIATPE----------PEVDVNQLSDSEVEAE-IERLLK
E.coli GETPSAVTRLSVVAKSEPQDEQSRSQSPRRIILSRLKAGEVDLLEEELGHLTTLTDVVKGADSLSAILP
H.pylori KRQEADKERRA-----QKKQEAKPKQEVTPTKETPKAPKTETKAKAKADTEENKAPSIGVEQTVRVDVR
C.jejuni VRKAEDQARRA-----QKKQTINAAPK--PTNNTANKPTESGEKKVPASGSNASS----MDQTIRVEVK
E.coli GDIAEDDITAVLCFVIEADQITFETVEVSPKISTPPVLKLAAEQAPTGRVEREKTTRSNESTSIRVAVE
P2
H.pylori RLDHLMNLIGELVLGKNRLIRIYSDVEERYDGEKFLEELNQVVSSISAVTTDLQLAVMKTRMQPVGKVF
C.jejuni RLDHLMNLIGELVLGKNRLLKIYDDVEERYEGEKFLEELNQVVSQLSIIITDVQLAVMKTRMQPIAKVF
E.coli KVDQLINLVGELVITQSMLAQRSSELDPVNHGD-----LITSMGQLQRNARDLQESVMSIRMMPMEYVF
H.pylori NKFPRMVRDLSRELGKSIELIIEGEETELDKSIVEEIGDPLIHIIRNSCDHGIEPLEERRKLNKPEIGK
C.jejuni NKFPRVVRDLSRELGKQIELEITGEETELDKSIVEEIGDPIMHMIRNSCDHGVEDPATRAANGKPEKGI
E.coli SRYPRLVRDLAGKLGKQVELTLVGSSTELDKSLIERIIDPLTHLVRNSLDHGIELPEKRLAAGKNSVGN
N**********N
H.pylori VQLSAYNEGNHIVIKISDDGKGLDPVMLKEKAIEKGVISERDAEGMSDREAFNLIFKPGFSTAKVVSNV
C.jejuni VQLKAYNEGNHIVVEITDDGKGLDPNGLKAKAIEKNLITEREADQMTDKEAFALIFKPGFSTAAKVTNV
E.coli LILSAEHQGGNICIEVTDDGAGLNRERILAKAASQGLTV---SENMSDDEVAMLIFAPGFSTAEQVTDV
G1********G1
F*********F
H.pylori SGRGVGMDVVKTNIEKLNGIIEIDSEVGVGTTQKLKIPLTLAIIQALLVGVQEEYYAIPLSSVLETVRI
C.jejuni SGRGVGMDVVKTNIEKLNGVIEIDSELGKGSSFKLKIPLTLAIIQSLLVGTQEEFYAIPLASVLETVRV
E.coli SGRGVGMDVVKRNIQKMGGHVEIQSKQGTGTTIRILLPLTLAILDGMSVRVADEVFILPLNAVMESLQP
G2**********G2
H.pylori SQDEIYTV-DGKSVLRLRDEVLSLVRLSDIFKVDAILESNSDVYVVIIGLADQKIGVIVDYLIGQEEVV
C.jejuni PIDDIYTI-EGKNVLRLRDEVLSLVRLSDVFGVKQVLESGDQTYVVVIGVAESKLGIIVDTLVGQEEIV
E.coli READLHPLAGGERVLEVRGEYLPIVELWKVFNVAGAKTEATQGIVVILQSGGRRYALLVDQLIGQHQVV

```
                    630         640         650         660
                     |           |           |           |
                |•||    ||  ••||••| ••• ||••|••|•|  | |  ||   |
H. pylori       IKSLGYYLKNTRGIAGATVRGDGKITLIVDVGAMMDMAKSIKVNITTLMN
C. jejuni       IKSMGDYLQNIQGIAGATIRGDGRVTLIIDVGAMMDMAKEIKVDIKAQL
E. coli         VKNLESNYRKVPGISAATILGDGSVALIVDVSALQAINREQRMANTA--A
```

3.2.1.10 Cloning and analysis of the *cheY* from *H. pylori* 26695

Specific primers, SPF1/2, flanking the *cheY* portion of *cheAY* (HP0392) (Tomb *et al* 1997) were synthesised incorporating *Pst*I and *Hind*III sites to amplify the second *cheY* gene from *H. pylori* chromosomal DNA. The 299 bp product was cloned into *Pst*I/*Hind*III digested pUC19 vector and designated pCYF. The four residues found in all CheY proteins to date, Asp12, Asp 13, Asp 57 and Lys 109 are conserved in the CheY domain of CheAY (Fig. 3.9). BLAST X searches revealed that the highest identity was to the N-terminus of bifunctional CheAY proteins from *S. coelicolor* and *P. aeruginosa* (40%). In this thesis the CheY of the bifunctional CheAY is designated CheY whereas the distinct CheY is referred to as CheY1.

Figure 3.9

Nucleotide and deduced amino acid sequence of the CheY domain from CheAY (HP0392) of *H. pylori* 26695. Residues of functional significance are indicated by bold lettering.

```
ATT GTC TTA GCG ATT GAT GAC AGC AGC ACG GAT AGA GCG ATT ATC CGC AAA TGT TTA AAA
I   V   L   A   I   D   D   S   S   T   D   R   A   I   I   R   K   C   L   K

CCA TTA GGC ATC ACG CTT TTA GAG GCG ACT AAC GGG TTA GAG GGC TTA GAA ATG CTT AAA
P   L   G   I   T   L   L   E   A   T   N   G   L   E   G   L   E   M   L   K

AAT GGC GAT AAG ATT CCG GAC GCT ATT TTA GTG GAT ATT GAA ATG CCT AAA ATG GAC GGC
N   G   D   K   I   P   D   A   I   L   V   D   I   E   M   P   K   M   D   G

TAC ACT TTC GCT TCT GAA GTG CGT AAA TAC AAT AAA TTC AAA AAC CTG CCT TTG ATC GCA
Y   T   F   A   S   E   V   R   K   Y   N   K   F   K   N   L   P   L   I   A

GTA ACC AGT CGG GTA ACT AAA ACC GAT AGG ATG CGT GGC GTT GAA TCC GGC ATG ACT GAA
V   T   S   R   V   T   K   T   D   R   M   R   G   V   E   S   G   M   T   E

TAC ATC ACC AAA CCT TAT AGC GGT GAA TAT TTA ACC ACC GTA GTG AAG CGC AGC ATT AAA
Y   I   T   K   P   Y   S   G   E   Y   L   T   T   V   V   K   R   S   I   K

TTA GAA GGA GAC CAA TCG
 L   E   G   D   Q   S
```

3.2.1.11 Conservation between CheY and CheY1

H. pylori CheY1 and CheY deduced amino acid sequences were aligned with *E. coli* and *C. jejuni* CheY sequences (Fig 3.10). The percentage homology between the CheY proteins from these three species is presented in Table 3.2.

Figure 3.10 Alignment of CheY proteins from *E. coli* (CheY E.c), *H. pylori* (CheY H.p and CheY1 H.p) and *C. jejuni* (CheY C.j and CheY1 C.j). Asterisks indicate putative residues involved in signalling to the flagellar motor switch complex. Sequences were aligned using Clustal V multiple alignment software.

```
                  10        20        30        40        50        60
                   |        |         |         |         |         |
                  |• |••    • ||||  •  ••   | •• |•||| | |         || • |•• •|•
CheY  E.c  MADKELKFLVVDDFSTMRRIVRNLLKELGFNNVEEAEDGVDALNKLQA-GGYGFVISDWNMPNMDGLE
CheY1 H.p  M-----KLLVVDDSSTMRRIIKNTLSRLGYEDVLEAEHGVEAWEKLDANADTKVLITDWNMPEMNGLD
CheY1 C.j  M-----KLLVVDDSSTMRRIIKNTLTRLGHDDVLEAEHGVEAWDLLTKNEDVKVLITDWNMPEMNGLE
CheY  H.p  ------IVLAIDDSSTDRAIIRKCLKPLGITLLEATNGLEGLEMLKNGDKIPDAILVDIEMPKMDGYT
CheY  C.j  ------KVLIVDDSKMDRTLMQKALEPLGVSLIEATNGVEALNIIKSGEHDIDAMLIDIEMPRMDGYT

           70        80        90        100       110       120       130
            |         |         |         |         |         |         |
            |   |•       |•|| |•|   •  | |  |   •   •| ••||   •   |   |
CheY  E.c  LLKTIRADGAMSALPVLMVTAEAKKENIIAAAQAGASGYVVKPFTPATLEEKLNKIF-----EKLGM
CheY1 H.p  LVKKVRSDSRFKEIPIIMITTEGGKAEVITALKAGVNNYIVKPFTPQVLKEKLEVVLGIN------D
CheY1 C.j  LVKKVRAEKKYEDMPIIMVTTEGGKAEVITALKAGVNNYIVKPFTPQVLKEKLEDVLGIGSGEGAAE
CheY  H.p  FASEVRKYNKFKNLPLIAVTSRVTKTDRMRGVESGMTEYITKPYSGEYLTTVVKRSIKLEGDQS
CheY  C.j  LAGEIRKYSKYRNLPLIAVTSRTSKTDRLRGVEVGMTEYITKPYSPEYLENVVRKNLKL----G
                          *         *    *   * **** **
```

Table 3.2 Percentage homology of the CheY proteins from *H. pylori, C. jejuni* and *E. coli*.

% Homology	H.p CheY	H.p CheY1
H.p CheY	100	34
H.p CheY1	34	100
C.j CheY	65	31
C.j CheY1	33	81
E.c CheY	30	49

3.3 Discussion

A *cheY* homologue was amplified from *H. pylori* NCTC11637 with degenerate primers (PCRDOP). The amino acid sequence of CheY proteins possess a high degree of conservation within their N-terminal domains, maintaining the functionally important site of phosphorylation. The design of degenerate primers is based on these conserved regions. A classical way to isolate homologous *cheY* genes would be by Southern hybridisation using gene probes derived from other enteric *cheY* genes (*E. coli*, *S. typhimurium*). However, *H. pylori* has a high (67%) AT content, which can significantly reduce the chances of hybridisation with genes from other species of bacteria even under conditions of low stringency. Additionally even if the probe did hybridise to a fragment of genomic *H. pylori* DNA, it may not be possible to clone the fragment if it is large or contains a toxic gene, which may prove lethal if expressed in *E. coli*. Consequently, attempts to isolate the *cheY* response regulator from *H. pylori* focused on the application of PCR with degenerate primers (PCRDOP). Previously PCRDOP has been shown to amplify response regulator gene fragments from a range of bacterial species (Wren *et al* 1992). PCRDOP successfully amplified the *cheY*1 gene fragment from *H. pylori*. Analysis of the derived nucleotide sequence revealed an internal *Hind*III site (AAGCTT) located 82 bp downstream of the forward PCRDOP primer. Given that the amplified DNA was digested with *Hind*III and *Pst*I, it is surprising that the 330 bp *cheY*1 PCRDOP product was cloned. The choice of enzymes incorporated into the 5' end of the degenerate primers may explain why only one of the six response regulators present in the annotated *H. pylori* genome (Tomb *et al* 1997) was isolated. Blunt end cloning, using PCRDOP products treated with T4 polymerase, would avoid the loss of any portion of the isolated gene fragment. However, analysis of the genome sequence found that although some of the other five response regulators (HP0116, HP0703, HP1021, HP1043, HP1365) contain internal *Hind*III and *Pst*I sites, none of them contain the

conserved regions used in any of the degenerate primer combinations (Table 3.1) for the PCRDOP reactions and therefore were never amplified.

The *cheY*1 from *H. pylori* encodes a protein of 124 amino acids. Comparative analysis of CheYs revealed the highest level of identity (81%) was found to a CheY isolated from *C. jejuni* (J. Marchant personal communication, Guerry *et al* 1996). Since this study commenced, two identical *H. pylori* CheY homologues have been published, from *H. pylori* CCUG17874 (99% identical) (Beier *et al* 1997) and *H. pylori* 26695 (HP1067) (98% identical) (Tomb *et al* 1997).

Multiple alignment of the six closest related CheY proteins, based on homology, from other bacterial species found that all structurally important regions (Asp 12, Asp 13, Asp 57 and Lys 109) were conserved in the CheY1 homologue from *H. pylori*.

Beier *et al* (1997) identified a 7137 bp operon which contains CheY1, a putative methyltransferase (*hsm*), the cell division protein, FtsH, the heavy metal binding proteins CopA and CopP, a putative membrane-associated phosphatidyltransferase, as well as an open reading frame (ORF) of unknown function, whose product is likely to be membrane-associated. This locus, designated the stress responsive operon (SRO), was found to be transcribed from a unique promoter upstream of *cheY*1. This promoter sequence, which was also identified in the nucleotide sequence of the *H. pylori* NCTC11637 *cheY*1 in this study, was found to be up regulated by high temperature and by the addition of copper ions to the culture media. The same gene organisation was found in *H. pylori* 26695 (HP1067-HP1073) (Tomb *et al* 1997). Partial sequencing downstream from the isolated *cheY*1 identified that the putative methyltransferase (*hsm*) was also conserved in *H. pylori* NCTC11637.

As outlined in Chapter 1, there are a variety of chemotaxis systems, with different combinations of chemotaxis-related homologues, found in bacterial species. *H. pylori* has a bi-functional CheAY protein (HP0392) (Tomb *et al* 1997), which

contains domains homologous to both CheA and CheY proteins, similar to the FrzE homologue found in the gliding bacterium *M. xanthus* (Acuna *et al* 1995). A high level of homology (65%) was found between the CheA domain of *H. pylori* and the closely related *C. jejuni*. Additionally database analysis revealed homology (38-42%) to a range of bacterial CheA's, particularly in functionally important regions, including *T. maritima*, *R. centenum* and *E. coli*, suggesting that *H. pylori* CheA probably also functions as a histidine protein kinase. The CheA region is followed by a 42 bp amino acid linker region (amino acid residues 664-677) which has no homology to any protein in the database. The CheAY hybrid of *M. xanthus* also has a linker region between the CheAY domains which exhibits no sequence similarity to that found in *H. pylori*. All of the previously identified critical residues involved in forming the acid pocket for acceptance of the phosphate from CheA are present in the CheY domain of CheAY. The highest identity was to the CheY domains of the CheAY homologues found in *C. jejuni* (65%), followed by *S. coelicolor* and *P. aeruginosa* (40%). This bifunctional protein structure is conserved throughout a range of species.

Other chemotaxis proteins identified by the genome sequencing project include a CheW homologue (HP0391), three CheV (CheW-Y) homologues (HP0019, HP0393, HP0616) and three genes homologous to classical methyl-accepting chemotaxis proteins (MCPs) (HP0099, HP0103 and HP0082). CheZ, CheB and CheR homologues found conserved in *E. coli* and *S. typhimurium* have not been identified in the *H. pylori* genome sequence. In Chapter 1, a description of the different chemotaxis homologues and how they function in different bacterial species ranging from those understood in detail, *E. coli* and *S. typhumurium*, to members of the α subgroup of *Proteobacteria*, demonstrates the complexity of chemotaxis systems studied to date (see section 1.7.8). The function of the chemotaxis homologues identified in *H. pylori* has not been determined. Of particular interest is the function of multiple copies of CheY proteins. The CheY1

and CheY proteins exhibit 49% and 30% identity respectively to the CheY sequence of *E. coli* and 34% similarity to each other. Additionally, a very similar configuration with two CheY proteins has also been identified in *C. jejuni.* Therefore it is unlikely that they are redundant versions of one CheY protein. In fact both *Helicobacter* and *Campylobacter* counterparts show high sequence similarities (82%) and (65%), suggesting that the CheY1 and CheY proteins were each derived from a common ancestral protein. Both CheY1 and CheY possess the phosphate accepting Asp-57 and the essential Asp-12, Asp 13 and Lys-109 residues as well as the hydrophobic core residues conserved among response regulator proteins (Stock *et al* 1993). These conserved sites suggest that both of these proteins function as response regulators. The conservation of two diverged proteins may suggest that these proteins have evolved vital functions. It is possible that the presence of two CheY homologues reflect two distinct transduction pathways, one using the MCP-CheA pathway, the other controlled by an alternative pathway, maybe linked to metabolic function. Alternatively both CheYs may interact with CheA. This is suggested by the difference between the P2 domain of *H. pylori* and *E. coli* CheA amino acid sequence.

An alternative explanation for the presence of two CheY homologues is suggested by the lack of a CheZ homologue in *H. pylori.* CheZ, which is also absent in other organisms, e.g *B. subtilis* and *R. centenum*, functions to catalyse the dephosphorylation of CheY-P (Hess *et al* 1988). The competition of phosphorylated CheY and CheY1 proteins for the switch complex in *H. pylori* could be a mechanism which would replace the requirement of CheZ. Scrutiny of the *Camplyobacter* genome sequence also reveals the absence of a CheZ homologue.

Only 2 of the 10 residues (Fig 3.10) which are thought to be involved in docking to the motor polypeptides, FliM and FliG (Sockett *et al* 1992), are conserved between *H. pylori* CheY1 and CheY. The motor docking domains were originally

identified by mutagenesis and crystal analysis of CheY homologues from *E. coli* and *S. typhimurium* (Roman *et al* 1992, Sockett *et al* 1992, Stock *et al* 1989 and Volz and Matsumura 1991). Their lack of conservation suggests that CheY1 and the CheY domain of CheAY may serve distinct roles in chemotactic signal transduction in *H. pylori.*

Subsequent chapters deal with the construction of defined mutants, and the identification of the roles played by the *cheY*, *cheY*1 and *cheA* in the motility, chemotaxis and virulence of *H. pylori.*

3.4 Summary

In this study homologues from three putative chemotaxis components were cloned from *H. pylori* NCTC11637. A CheY homologue, designated *cheY*1, was isolated using PCR with degenerate primers (PCRDOP). Two other components, a CheA domain and a second CheY domain of the bi-functional CheAY were also cloned. The CheA domain of CheAY was identified by random sequencing of a gene library. This clone (supplied by A. Labigne) was partially sequenced and a gene fragment cloned. The CheY domain of CheAY was cloned using information from *H. pylori* 26695 genome sequence, released in August 1997 (Tomb *et al* 1997). The nucleotide sequences of *cheY*1, *cheA* and *cheY* and analysis of the deduced amino acid sequences are presented in this chapter. The function of these and the other homologues identified in the genome sequence (Tomb *et al* 1997) have yet to be studied.

Chapter 4

Construction of defined *cheY*1, *cheAY*, *cheY* and *cheAY/Y*1 *H. pylori* N6 and SS1 mutants

4.1 Introduction

Three putative components of the *H. pylori* chemotaxis system (*cheY*1, *cheAY* and *cheY*) were identified, cloned and sequenced from *H. pylori* NCTC11637 (see Chapter 3). In other bacterial species, studies of *cheY* and *cheAY* deletion mutants have demonstrated that these genes are essential for functioning of a chemotaxis system (Manson *et al* 1998).

Inverse PCR mutagenesis (IPCRM) is a method which allows a precise deletion to be introduced into the cloned *H. pylori* gene and simultaneously engineers a unique restriction site for insertion of a selectable marker, such as an antibiotic resistance cassette (Wren *et al* 1994, Dorrell *et al* 1996). IPCRM utilises gene or gene fragments cloned into pUC19 as a circular template for an inverse PCR reaction. A single pair of inverse primers are designed to incorporate a defined deletion in the cloned gene or gene fragment (Fig. 4.1). The major advantage of this technique is that small motifs known to be essential for protein function can be specifically deleted. After IPCRM, the gene construct is introduced into *H. pylori* host cells by either natural transformation or electroporation. This pUC based construct contains an *E. coli* origin of replication and cannot be maintained in the *H. pylori* host cell. Selection for antibiotic resistant colonies facilitates the identification of *H. pylori* mutants where the mutated gene construct containing the antibiotic resistant cassette has recombined into the chromosomal gene locus. The aim of this chapter was the construction of defined *H. pylori* N6 and SS1 *cheY*1, *cheAY*, *cheY* and *cheAY/Y*1 isogenic mutants using the IPCRM and allelic exchange procedure depicted in Figure 4.1.

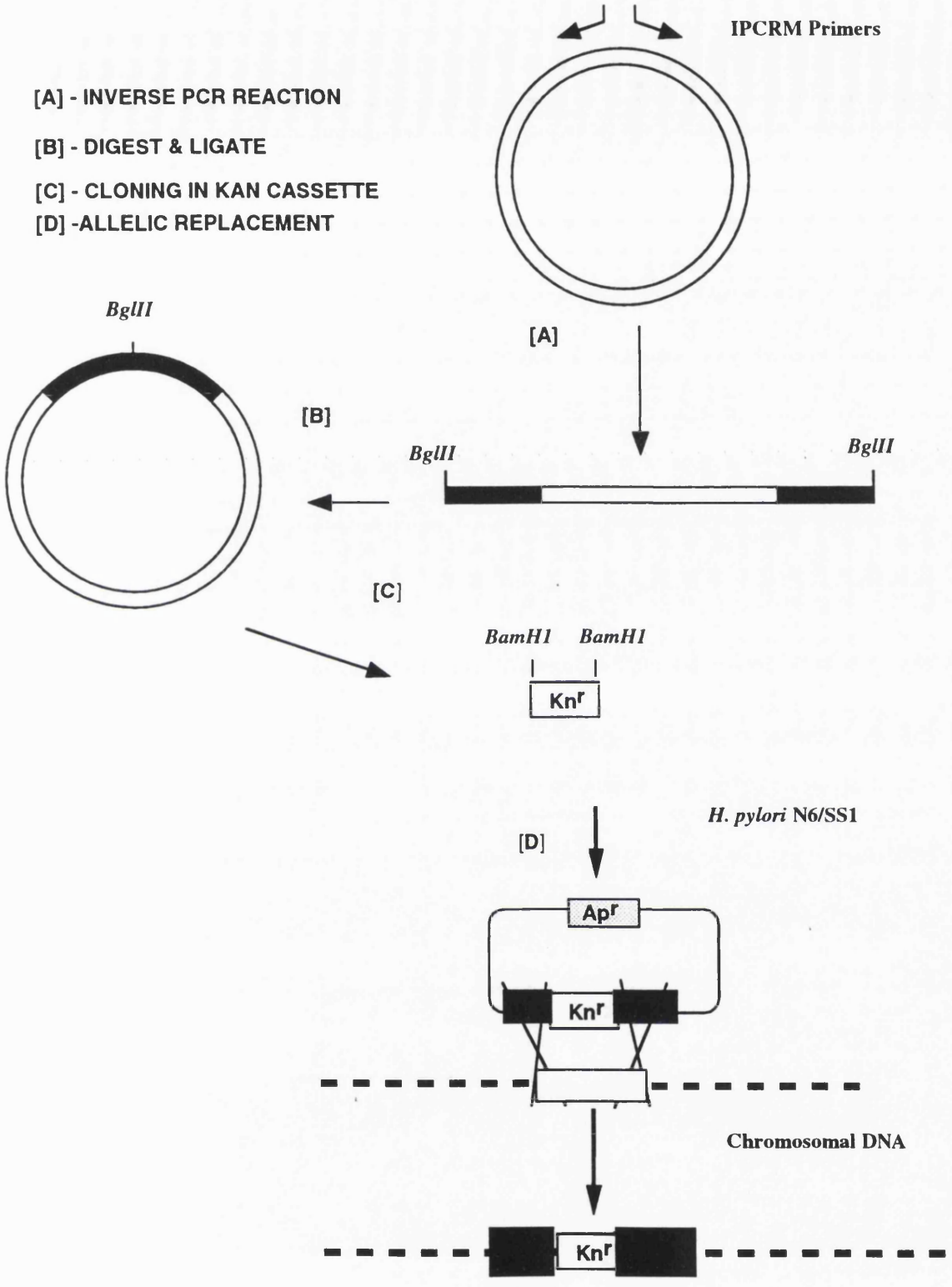


Figure 4.1. Procedure used for the construction of defined *H. pylori* chemotaxis mutants during this study.

4.2 Results

4.2.1 Construction of *H. pylori* N6 and SS1 *cheY*1 mutants

4.2.1.1 Generation of a *cheY*1 deletion construct by IPCRM

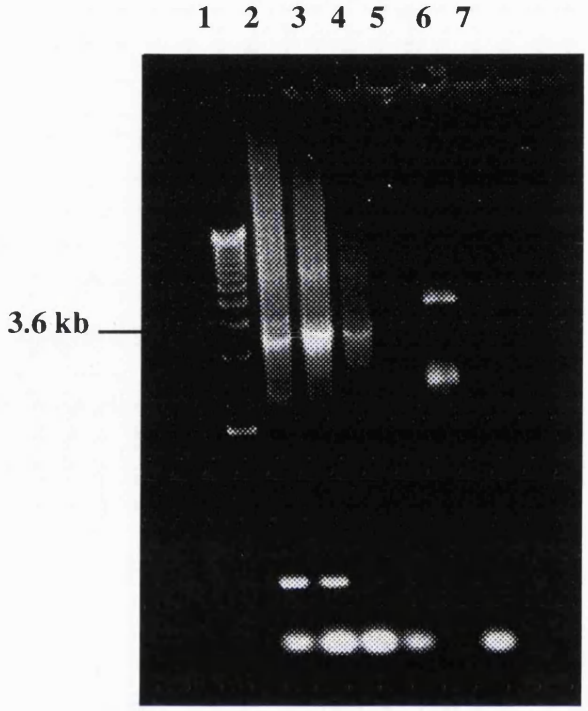
IPCRM using primers IPCY1 and IPCY2 (Table 2.4) each containing a 5' terminal *Bgl*II restriction site were used to introduce a 19 bp deletion and a *Bgl*II site into pCY110. The primers used for IPCRM were designed to delete a central portion of the *H. pylori* CheY1 which include three of the conserved sites, 66, 68 and 69 from the α 3 helices (Volz 1993). The resulting 3.6 kb amplified product (Fig 4.2a, lane 3) was digested with *Bgl*II, re-circularised by ligation and transformed into *E. coli* XL2 Blue MRF' cells as described in section 2.2.7. Transformants were screened by PCR using vector specific primers, V1 & V2, to size select the putative IPCRM clones as described in section 2.2.8. As the 19 bp deletion introduced by IPCRM was not large enough to be visualised on an agarose gel, the PCR products were digested with *Bgl*II to select clones which contained a *Bgl*II site. Three clones analysed (pCYIP3, pCYIP4 and pCYIP5, Fig 4.2b) produced two products when digested with *Bgl*II, confirming the presence of the introduced *Bgl*II site and suggesting the incorporation of the defined deletion. For confirmation of the defined deletion, pCYIP3 was sequenced using primer SPCY3, which anneals 200 bp upstream from the engineered *Bgl*II site. Analysis of the sequence data revealed that the insert contained the expected deletion and a unique *Bgl*II site (Fig. 4.3).

Figure 4.2

(a) Introduction of defined deletion and unique *Bgl*II site into pCY110 by IPCRM using primers IPCY1 & IPCY2. Lane 1, 1 kb molecular weight marker; lane 2, 1 µl template; lane 3, 0.1 µl template; lane 4, 0.01 µl template; lane 5, 0.001 µl template; lane 6, DNA template control, for comparison to amplified PCR product; lane 7, negative control.

(b) Digestion of V1 & V2 PCR products with *Bgl*II. Lane 1, 1 kb molecular weight marker; PCR products from pCYIP3-5 undigested (lanes 2, 4, 6); PCR products from pCYIP3-5 digested (lanes 3, 5, 7).

(a)



(b)

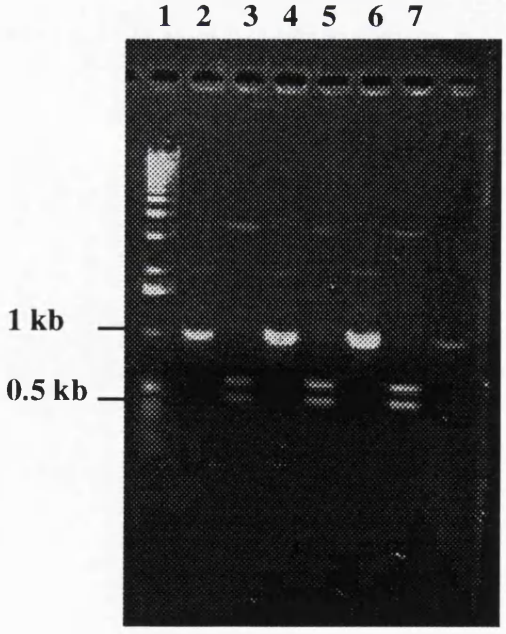


Figure 4.3

Introduction of a defined deletion and unique *Bgl*II site into pCY110 by IPCRM.

(a) Sequence of pCY110. Sequence deleted during IPCRM is shown in bold. **(b)** Sequence and orientation of the IPCRM primers. The *Bgl*II sites are underlined.

(c) Construct pCY3 (confirmed by DNA sequencing)

(a)

```
5' AAC ATG CCT GAA ATG AAC GGC TTA GAT CTC GTT AAA AAG GTG CGC TCC GAT AGC CGC 3'
   N   M   P   E   M   N   G   L   D   L   V   K   K   V   R   S   D   S   R
```

(b)

```
                                        5' CG AGATCT AAG GTG CGC TCC GAT AGC3'
                                                   K   V   R   S   D   S
                                                        PrimerIPCY2

3' TAC GGA CTT TAC TTG TCTAGA GA 5'
   M   P   E   M   N
       Primer IPCY1
```

IPCRM and self-ligation

(c)

```
5' AAC ATG CCT GAA ATG AAC AGATCT AG GTG CGC TCC GAT AGC CGC 3'
   N   M   P   E   M   N  BglII     V   R   S   D   S   R
```

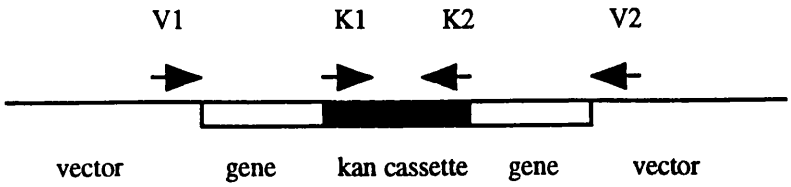


Figure 4.4c: Positions of primers VI and V2 and K1 and K2 used to determine the orientation of the kanamycin cassette cloned into mutated IPCRM constructs throughout the project

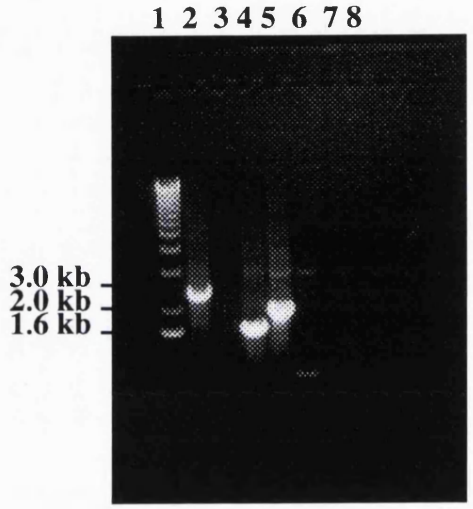
4.2.1.2 Insertion of a kanamycin cassette into pCYIP3

A *Bam*HI restriction fragment of pJMK30 (J. M. Ketley) containing a 1.4 kb kanamycin resistance gene (*aph3'*-III) (Ferrero *et al* 1992) was cloned into the unique *Bgl*II site of pCYIP3 and transformed into *E. coli* XL2 Blue MRF' cells. The resultant kanamycin resistant transformants were analysed by PCR using vector specific primers, V1 & V2, to confirm the insertion of the kanamycin cassette (Fig 4.4, lane 2). Two clones found to contain the correct insert size of 2.4 kb (insert 1 kb + kanamycin cassette 1.4 kb) were analysed further by PCR using a combination of vector specific primers, V1 & V2, and kanamycin specific primers, K1 & K2, (see Figure 4.4c) to determine the orientation of the kanamycin cassette in relation to the direction of translation of the *cheY*1. Figure 4.4a shows that in clone pCYIPk1 the kanamycin cassette was cloned in the same orientation as the direction of translation of the *cheY*1 (lanes 3-6). By contrast, the plasmid pCYIPk2 contained the kanamycin cassette in the opposite orientation (Fig.4.4b; lanes 3-6) .

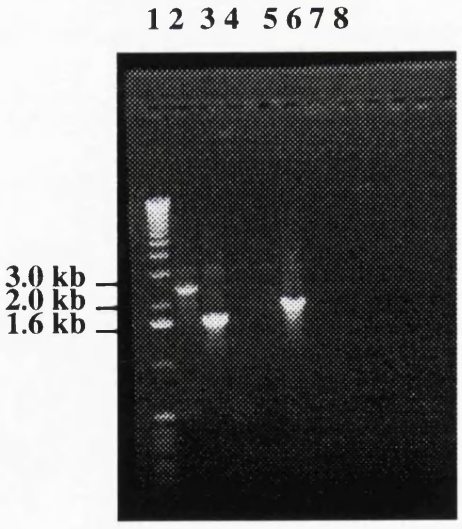
Figure 4.4

PCR analysis of pCYIPk1 **(a)** and pCYIPk2 **(b)**, generated by the insertion of the kanamycin resistance gene into the unique *Bgl*II site of pCYIP3, using a combination of vector specific primers,V1 & V2 , expected product of 2.4 kb (1 kb gene+ 1.4 kb kanamycin casette) and kanamycin specific primers, K1 & K2, expected product 1.4 kb. See Figure 4.4c. Both gels show the same primer combinations in each lane. Lane 1, 1 kb molecular weight marker; lane 2, V1 & V2; lane 3, V1 & K1; lane 4, V1 & K2; lane 5, V2 & K1; lane 6, V2 & K2; lane 7, V1 & V2 negative control; lane 8, K1 & K2 negative control. Negative controls contained PCR mix with primers but without template.

(a)



(b)



4.2.1.3 Mutagenesis of *cheY1* in *H. pylori* N6 and SS1 (ΔCheY1⁻).

Plasmids pCYIPk1 and pCYIPk2 were introduced into *H. pylori* N6 by natural transformation as described in section 2.5.2. No transformants were obtained using construct pCYIPk1. In contrast, a transformation frequency of $1x10^5$ kanamycin resistant (Km^r) transformants per μg of DNA was observed using construct pCYIPk2. Putative mutants selected on blood agar plates containing kanamycin (25 μg/ml) were screened by PCR to confirm that the transformants resulted from allelic replacement rather than plasmid integration. Genomic DNA isolated from the Km^r transformants CYM1, CYM2 and from the parental strain N6 were analysed by PCR using *cheY1* specific primers SPCY3 and SPCY4. These primers amplified a 1.8 kb product from the mutant strains CYM1 and CYM2 consistant with the insertion of a 1.4 kb kanamycin resistance cassette into the wild type gene. As expected a 0.4 kb product was amplified when these same primers were used with the parental N6 strain as the template (Fig. 4.5).

Southern hybridisation analysis (as described in section 2.3) of *Hae*III digested genomic DNA from a range of putative *cheY1* mutants, CYM1 to CYM7, provided further confirmation of the allelic replacement event. A 1 kb probe (PCR product amplified from pCY110 using primers SPCY1 & SPCY2) containing *cheY1*, hybridised to a 1.4 kb *Hae*III fragment in the parental N6 strain and to two fragments of 1.8 kb and 1.0 kb in all the putative *cheY1* mutants tested. This result is consistent with the insertion of a 1.4 kb kanamycin cassette into the wild type *cheY1* (Fig. 4.6). Two fragments were found to hybridise to the probe in the mutated strains as the kanamycin cassette contains two *Hae*III sites at nucleotide positions 880 and 1057. Digestion with *Hae*III results in three fragments, however one of these at approximately 180 bp in size would not have been visible on this gel prior to blotting. This construct was also used to generate a *cheY1* mutant in the *H. pylori* SS1 strain via electroporation. Successful mutagenesis was

confirmed by PCR (data not shown). The *cheY*1 mutant in SS1 was termed RAS1 (SS1ΔCheY1$^-$).

Figure 4.5

PCR analysis of *H. pylori* N6 *cheY*1 mutants (N6ΔCheY1$^-$). Genomic DNA was analysed by PCR using the *cheY*1 specific primers SPCY3 & SPCY4. Lane 1, 1 kb molecular weight markers; lane 2, N6 wild type; lane 3 & 4, putative *cheY*1 mutants CYM1 and CYM2; lane 5, positive control; pCYIPk2; lane 6, negative control. Negative controls contained PCR mix with primers but without template.

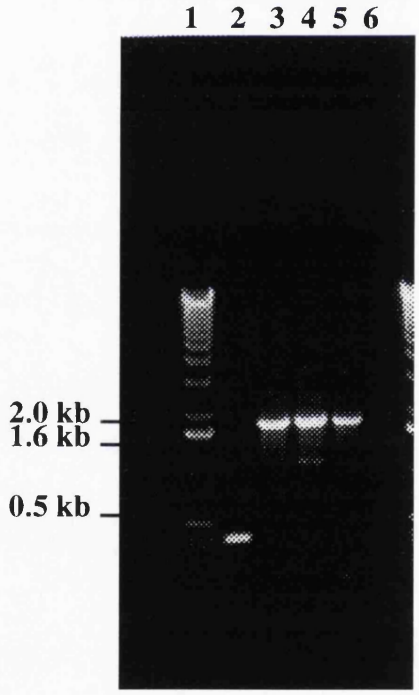
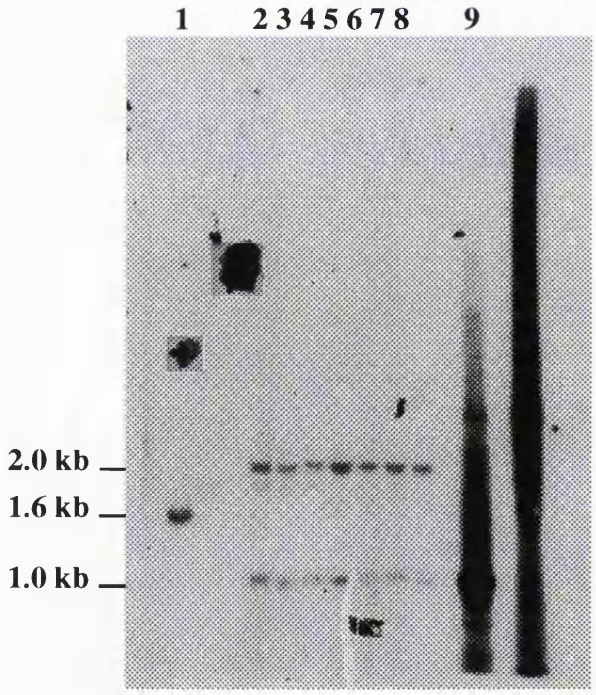


Figure 4.6

Southern hybridisation analysis of *H. pylori* N6 *cheY*1 mutants (ΔCheY1$^-$). *Hae*III digested genomic DNA was hybridised using a 1 kb fragment containing *cheY*1 as a probe. Lane 1, N6; lanes 2-8; putative mutants CYM1-CYM7; lane 9; 1 kb *Hind*III/*Pst*I fragment which contains *cheY*1 as a positive control. Lane 10 contains kanamycin cassette, which acted as a positive control in a subsequent Southern blot hybridised with the kanamycin cassette (data not shown).



4.2.2 Construction of *H. pylori* N6 and SS1*cheAY* mutants.

4.2.2.1 Generation of a *cheAY* deletion construct by IPCRM

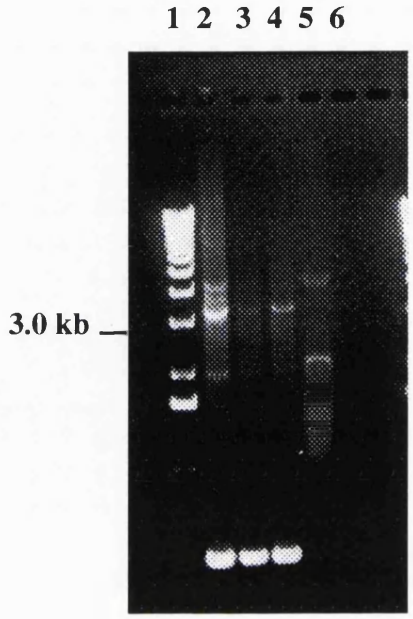
IPCRM using primers IPCA1 & IPCA2 was used to introduce a *Bgl*II site and a 37 bp deletion into pCA110 (Fig. 4.7a). This 37 bp deletion was in the C-terminal portion of the CheA domain (Stock *et al* 1995). The resulting 3.4 kb product was column purified, digested with *Bgl*II, circularised by ligation and transformed into *E. coli* XL2 Blue MRF' as described in section 2.2.7. Transformants were screened by PCR using vector specific primers and the resulting products were digested with *Bgl*II (as described in section 4.2.1.1). Three clones analysed (pCAIP2, pCAIP4 and pCAIP5) all contained an internal *Bgl*II site and were also assumed to contain the defined deletion (Fig. 4.7b). This was confirmed for clone pCAIP2 by sequencing, using primer C3.4, which anneals 60 bp upstream from the deletion (Fig. 4.8).

Figure 4.7

(a) Introduction of a defined deletion and a unique *Bgl*II site into pCA110 using IPCRM with primers IPCA1 & IPCA2. Lane 1, 1 kb molecular weight marker; lane 2, 1 µl template; lane 3, 0.1 µl template; lane 4, 0.01 µl template; lane 5, DNA template control, for comparison to amplified PCR product; lane 6, negative control. Negative controls contained PCR mix with primers but without template.

(b) Digestion of V1 & V2 PCR products with *Bgl*II. Lane 1, 1 kb molecular weight marker. PCR products from pCAIP2, pCAIP4, pCAIP5 and pCA110 undigested (lanes 2, 4, 6, 8); PCR products from pCAIP2, pCAIP4, pCAIP5 and pCA110 digested (lanes 3, 5, 7, 9); lane 10, negative control. Negative controls contained PCR mix with primers but without template.

(a)



(b)

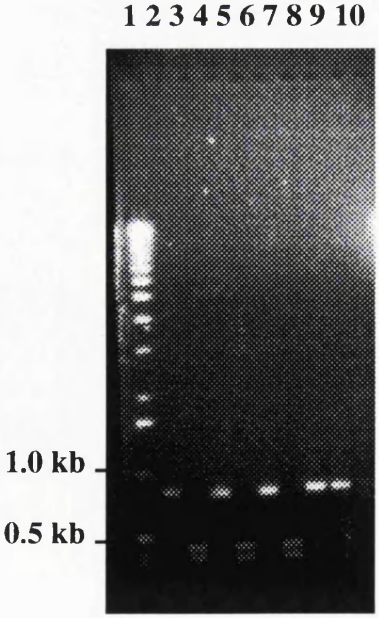
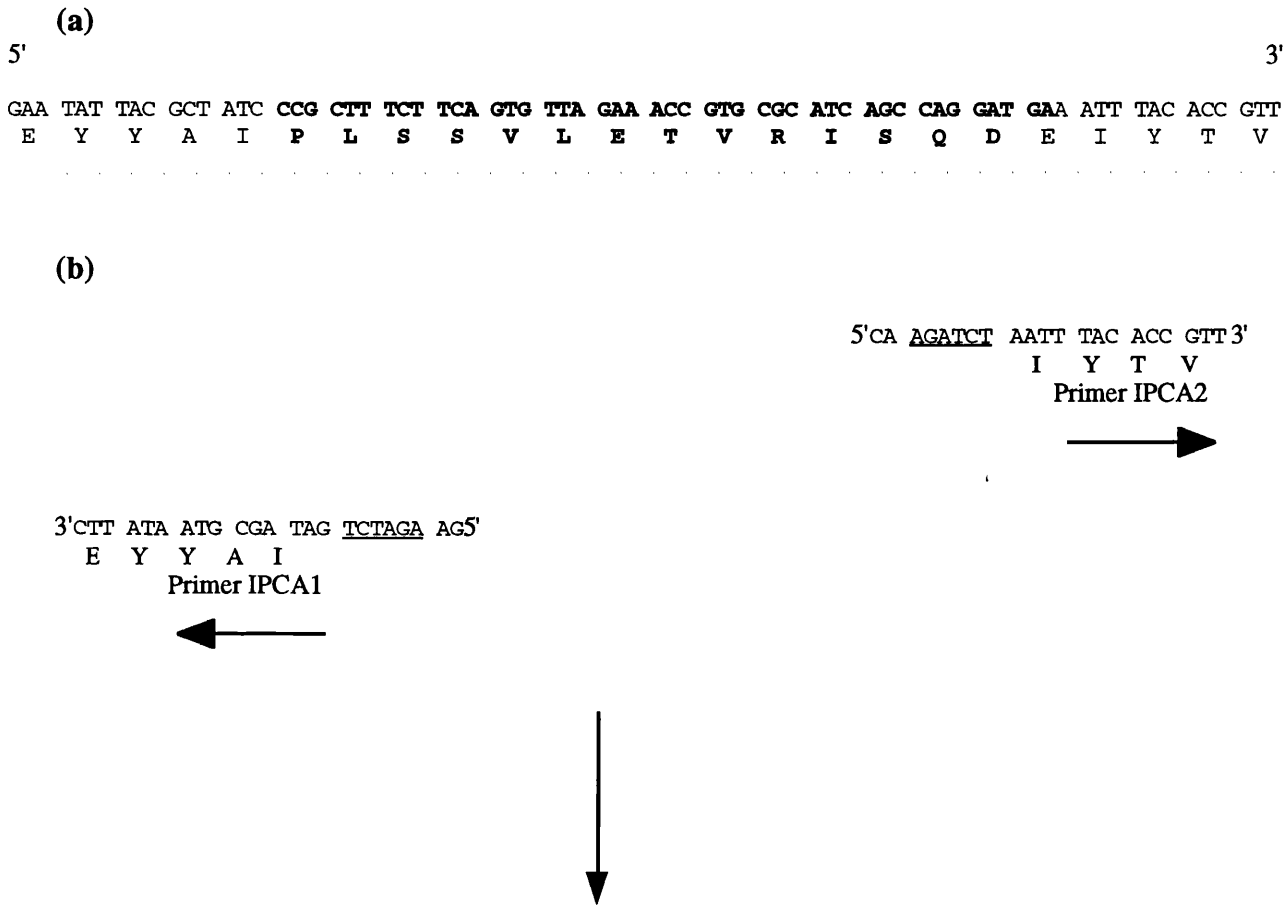


Figure 4.8

Introduction of a defined deletion and unique *Bgl*II site into pCA110 by IPCRM. **(a)** Sequence of pCA110. Sequence deleted during IPCRM is shown in bold. **(b)** Sequence and orientation of the IPCRM primers. The *Bgl*II sites are underlined. **(c)** Construct pCAIP2 (confirmed by DNA sequencing).

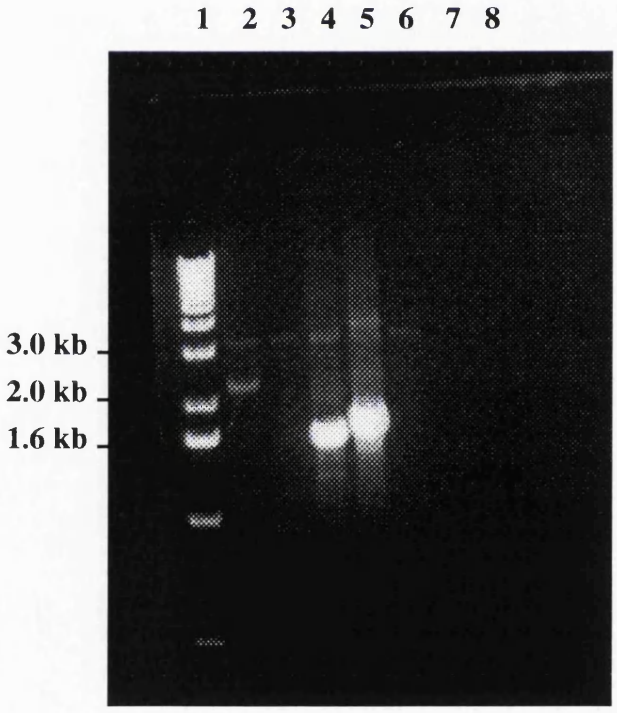


4.2.2.2 Insertion of a kanamycin cassette into pCAIP2.

Plasmid pCAIP2 was digested with *Bgl*II and ligated with a *Bam*HI restriction fragment of pJMK30 containing the kanamycin cassette and transformed into *E. coli* XL2 Blue MRF' cells. To identify those clones which contained the kanamycin cassette, the kanamycin resistant transformants were screened by PCR using vector specific primers, V1 & V2 as described in section 4.2.1.1 (Fig 4.9, lane 2). One clone, pCAIPk1, was analysed further to determine the orientation of the kanamycin cassette (as described in section 4.2.1.2, See Figure 4.4c). The results of PCR experiments using combinations of vector and kanamycin specific primers indicate that pCAIPk1 contains the kanamycin cassette in the forward orientation (Fig 4.9).

Figure 4.9

PCR analysis of pCAIPk1, generated by the insertion of the kanamycin resistance gene into the unique *Bgl*II site of pIPCA2, using a combination of vector specific primers, V1 & V2, expected product of 2.2 kb (0.8 kb gene fragment + 1.4 kb kanamycin casette) and kanamycin specific primers, K1 & K2, expected product 1.4 kb. Lane 1, 1 kb molecular weight marker; lane 2, V1 & V2; lane 3, V1 & K1; lane 4, V1 & K2; lane 5, V2 & K1; lane 6, V2 & K2; lane 7, V1 & V2 negative control; lane 8, K1 & K2 negative control. Negative controls contained PCR mix with primers but without template.



4.2.2.3 Mutagenesis of *cheAY* in *H. pylori* N6 and SS1 (ΔCheAY⁻).

Plasmid pCAIPk1 was transformed into *H. pylori* N6 by natural transformation as described in section 2.5.2 and a transformation frequency of $2x10^4$ kanamycin resistant (Km^r) transformants per mg of DNA was observed. Putative mutants were screened by PCR analysis of genomic DNA using specific *cheA* primers, SPCA1 & SPCA2. A product of 2.2 kb was amplified from both putative mutant strains CAM1 and CAM5, by contrast a product of 0.8 kb was amplified from the wild type N6 genomic DNA. This is consistent with the insertion of a 1.4 kb kanamycin cassette into the wild type *cheAY* (Fig. 4.10).

Allelic replacement of the *cheAY* wild type gene was confirmed by Southern hybridisation. Hybridisation was performed on *Hae*III digested genomic DNA using a 0.7 kb *Hind*III/*Pst*I fragment of pCA110 as a probe. The *cheA* probe hybridised to a 8.9 kb fragment in the wild type strain, and to two fragment of approximately 5.5 kb and 5 kb in both the *cheAY* mutants, CAM1 and CAM5, analysed (Fig. 4.11). This result confirmed successful allelic replacement of the wild type gene with the mutated *cheAY*. This construct was also used to generate the *cheAY* mutant in the *H. pylori* strain SS1 via electroporation as described in section 2.5.3. Successful mutagenesis was confirmed by PCR (data not shown). The *cheAY* mutant in SS1 was termed SF6 (ΔCheAY⁻).

Figure 4.10

PCR analysis of *H. pylori* N6 *cheA* mutants. Genomic DNA was analysed by specific *cheA* primers SPCA1 & SPCA2. Lane 1, 1 kb molecular weight markers, lane 2, N6; lane 3, 4, putative *cheAY* mutants, CAM1, CAM5; lane 5, pIPCAK1; lane 6, negative control as before.

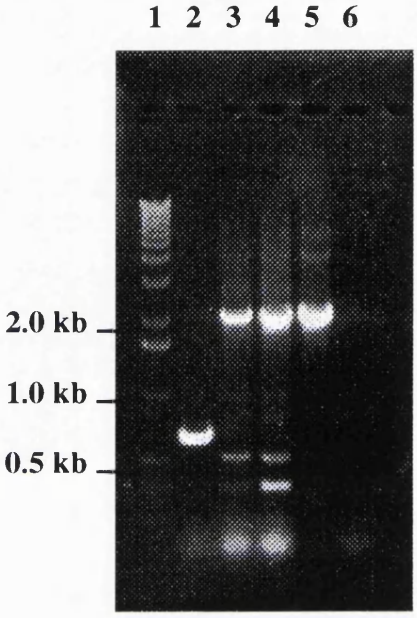
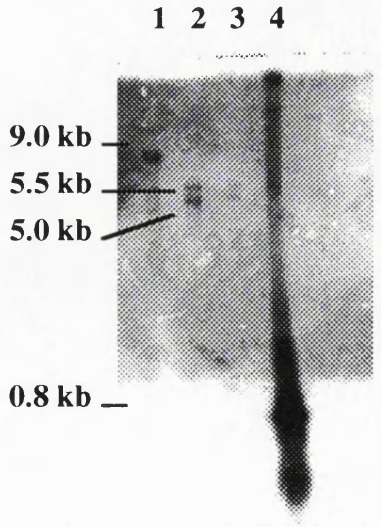


Figure 4.11 Southern hybridisation analysis of *H. pylori* N6 *cheAY* mutants. *Hae*III digested genomic DNA was hybridised using a 0.8 kb fragment of the CheA domain as a probe. Lane 1, N6; lanes 2 & 3; putative mutants CAM1 and CAM7; lane 4, 0.8 kb *Hind*III/*Pst*I fragment of pCA110 which acts as a positive control.



4.2.3 Construction of a *H. pylori* N6 *cheY* mutant.

4.2.3.1 Generation of a *cheY* deletion construct by IPCRM

IPCRM using primers IPCF1 & IPCF2 was used to introduce a *Bgl*II site and a 5 bp deletion into pCF (Fig 4.12a). The resulting 3 kb product was digested with *Bgl*II, religated and transformed into *E. coli* XL2 Blue MRF' cells as described insection 2.2.7. Transformants were analysed as described in sections 4.2.1.1 and 4.2.2.1 and those which contained a *Bgl*II site were also assumed to contain the defined deletion. One clone pCFIP1 was selected for further study (Fig 4.12b). The alteration in the nucleotide sequence as a result of IPCRM of clone pCF is shown in Fig. 4.13.

Figure 4.12

(a) Introduction of a 5 bp deletion and a unique *Bgl*II site into pCF using IPCRM with primers IPCF1 & IPCF2. Lane 1, 1 kb molecular weight marker; lane 2, 1 µl template; lane 3, 0.1 µl template; lane 4, 0.01 µl template; lane 5, DNA template control (as Figure 4.7); lane 6, negative control as before.

(b) Digestion of V1 & V2 PCR products with *Bgl*II. Lane 1, 1 kb molecular weight marker. PCR products from pCFIP1, pCFIP4, pCFIP5 and pCF undigested (even lanes 2, 4, 6, 8); PCR products from pCFIP1, pCFIP4, pCFIP5 and pCF digested (odd lanes 3, 5, 7, 9); lane 10, negative control as before.

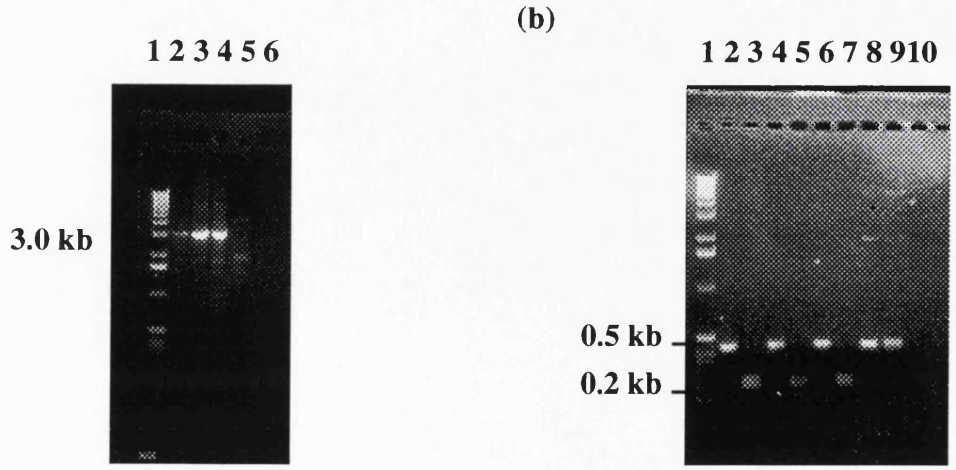


Figure 4.13

Introduction of a defined deletion and a *Bgl*II site into pCF by IPCRM.

(a) Sequence of pCF. Sequence deleted during IPCRM is shown in bold. **(b)** Sequence and orientation of the IPCRM primers. The *Bgl*II sites are underlined. **(c)** Construct pCFIP1 (confirmed by DNA sequencing)

(a)

5'CGT AAA TAC AAT AAA TTC **AAA AAC** CTG CCT TTG ATC GCA 3'
R K Y N K F **K** N L P L I A

(b)

5'TA AGATCT C CTG CCT TTG ATC GC 3'
L P L Y A
PrimerIPCF2

3'T TTA TGT TAT TTA AG TCTAGA AG5'
F K N Y K
Primer IPCF1

IPCRM and self-ligation

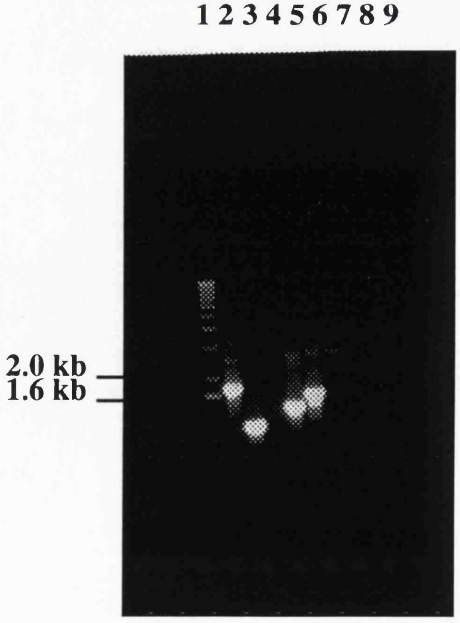
(c)

5'CGT AAA TAC AAT AAA TTC AGATCT C CTG CCT TTG ATC GCA 3'
R K Y N K F *Bgl*II L P L I A

4.2.3.2 Insertion of a kanamycin cassette into pCFIP

As in section 4.2.1.2 and 4.2.2.2, a *Bam*H1 digested kanamycin cassette was cloned into the *Bgl*II digested construct, pCFIP1, and transformed into *E. coli* XL2 Blue MRF' cells. Putative mutant constructs were screened, as described in section 4.2.1.2 and 4.2.2.2, using a combination of vector and kanamycin cassette specific primers to identify those clones which contained the kanamycin cassette and in which orientation (See Figure 4.4c). The results (Fig. 4.14, lanes; 4-7) indicate that pCFIPk1 contains the kanamycin cassette in the forward orientation.

Figure 4.14.PCR analysis of the plasmid pCFIPk1, generated by the insertion of the kanamycin resistance gene into the unique *Bgl*II site of pCFIP1, using a combination of vector specific primers, V1 & V2, expected product of 1.7 kb (0.3 kb gene fragment + 1.4 kb kanamycin casette) and kanamycin specific primers, K1 & K2. Lane 1, 1 kb molecular weight marker; lane 2, V1 & V2; lane 3, K1 & K2; lane 4, V1 & K1; lane 5, V1 & K2; lane 6, V2 & K1; lane 7, V2 & K2; lane 8, V1 & V2 negative control; lane 9, K1 & K2 negative control. Negative controls as before.

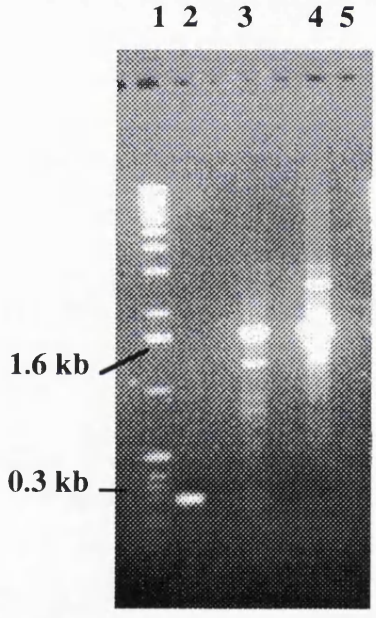


4.2.3.3 Mutagenesis of *cheY* in *H. pylori* N6 (ΔCheY⁻).

The mutated construct, pCFIPk1, was electrophorated into *H. pylori* N6 at a transformation frequency of $1x10^5$ kanamycin resistant (Km^r) transformants per μg of DNA. Putative mutants were screened by PCR analysis of genomic DNA using specific primers SPCF1 & SPCF2. A product of 1.7 kb was amplified from both the putative mutant strain CYFM1 and from the mutated gene construct, pCFIPk1, which acts as a positive control. As expected a product of 300 bp was amplified from the wild type N6 genomic DNA (Fig. 4.15). As before, Southern hybridisation was used to confirm the PCR results. *Hae*III digested chromosomal DNA from CYFM1 and N6 was hybridised to a *cheY* probe (*Hind*III/*Pst*I digest of pCF1). A single hybridisation product of approximately 9 kb was identified in the wild type N6 chromosomal DNA. In contrast two products of approximately 4 kb and 6.5 kb were identified in the mutated chromosomal DNA from CYFM1 (data not shown). This confirmed that the kanamycin cassette had been successfully inserted into *cheY* in *H. pylori* N6.

Figure 4.15

PCR analysis of *H. pylori* N6 *cheY* mutants. Genomic DNA was analysed by specific *cheY* primers SPCF1 & SPCF2. Lane 1, 1 kb molecular weight markers; lane 2, N6; lane 3, putative *cheY*1 mutant, CYFM1; lane 4, pCFIPk1; lane 5, negative control. The 2.7 kb and 3 kb bands visible in lane 4 is plasmid template from putative pCFIPk1 clones. Negative controls as before.



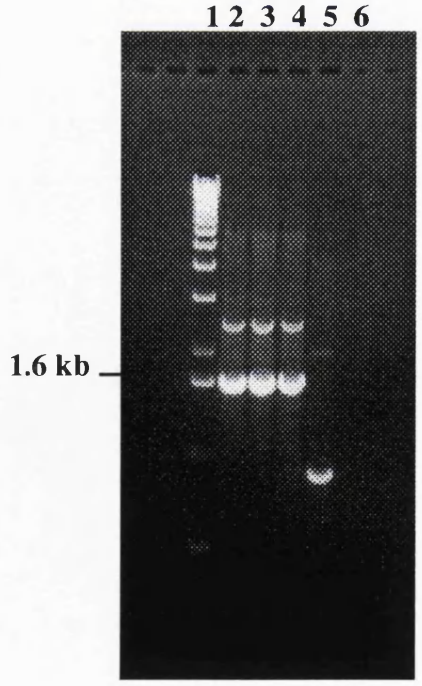
4.2.4 Construction of a *H. pylori* N6 *cheAY/Y1* mutant.

4.2.4.1 Insertion of a chloramphenicol cassette into pCAIP2

The chloramphenicol cassette, originally isolated from *C. coli* (Wang *et al* 1990), was used for the construction of a double *cheAY/Y1* mutant. pCAIP2 was digested with *Bgl*II and treated with Mung Bean nuclease to generate blunt ended plasmid as described in section 2.2.3. The 0.8 kb chloramphenicol cassette was obtained by *Hinc*II digestion of pCAT (C. Clayton). Following ligation and transformation, into *E. coli* XL1 Blue MRF' cells, chloramphenicol resistant clones where analysed using vector specific primers (V1 & V2) to identify those clones which contained a 0.8 kb increase in insert size. Figure 4.16 confirms that the *cheA*+CAT containing plasmid, pCAIPc, has been successfully constructed.

Figure 4.16

PCR analysis of pCAIPc, generated by the insertion of the chloramphenicol resistance cassette into blunt-ended *Bgl*II digested pCAIP2, using vector specific primers, V1 & V2, expected size 0.8 kb cheA gene fragment + 0.8 kb CAT cassette. Lane 1, 1 kb molecular weight marker; lanes 2, 3 and 4, putative pCAIPc clones; lane 5, pCAIP2, positive control; lane 6, negative control. Negative controls contained PCR mix with primers but without template. The 2.5 kb band and faint 6 kb band visible in lanes 2, 3 and 4 is plasmid template from putative pCAIPc clones.



4.2.4.2 Mutagenesis and confirmation of both the *cheAY* and *cheY*1 mutant in *H. pylori* N6 (ΔCheAY/Y1⁻).

A double mutant was constructed by the introduction of a second selectable marker, chloramphenicol acetyl transferase (CAT) cassette, for the disruption of single allele mutants that were already kanamycin resistant. As outlined in sections 4.2.2.1 and 4.2.4.1, the *cheAY* gene was disrupted by IPCRM and a chloramphenicol cassette, digested by *Hinc*II, was cloned into the resulting construct to generate pCAIPc. Bacterial cells from a N6ΔCheY1⁻ mutant, CYM1, were transformed by electroporation with the construct pCAIPc, and the resulting putative double mutants were selected on selective *H. pylori* media (section 2.1) containing kanamycin (25 μg/ml) and chloramphenicol (6 μg/ml). Genomic DNA from a putative mutant, CAYM8, was screened using two sets of specific primers, *cheY*1 specific SPCY3 & SPCY4 and *cheA* specific SPCA1 & SPCA2. When CAYM8 was PCR amplified using SPCY3 & 4 (*cheY*1 specific), a product of 1.7 kb, 1.4 kb larger than the product of 0.3 kb amplified from the parental N6 strain was amplified. PCR analysis of the same genomic DNA using SPCA1 & 2 (*cheA* specific) gave an amplified product of 1.6 kb, 0.8 kb larger than the product of 0.8 kb amplified from the parental N6 strain using the same set of primers (Fig. 4.17). These results confirmed CAYM8 as a double mutant in which both the *cheY*1 and *cheAY* genes are interrupted by kanamycin and chloramphenicol antibiotic resistance cassettes respectively. Southern hybridisation confirmed allelic replacement of both *cheAY* and *cheY*1 in *H. pylori* N6. *Hae*III digested chromosomal DNA from the wildtype N6 and from two putative mutant strains, CAYM8 and CAYM6, were analysed by hybridisation studies to specific *cheY*1 and *cheA* probes (probes as in sections 4.2.1.3 and 4.2.2.3 respectively). The results of the hybridisation using the *cheY*1 probe (Fig 4.18a) indicate that the *cheY*1 has been successfully interrupted by the kanamycin cassette. In both double mutants tested (CAYM6 and CAYM8) two fragments of 1.8 kb and 1.0 kb

hybridised to the *cheY*1 probe, in contrast a fragment of approximately 1.4 kb from the wild type N6 hybridised to the same probe. This 1.4 kb difference represents the presence of the kanamycin cassette in the putative double mutants CAYM6 and CAYM8. The *cheY*1 probe also hybridised to the 1 kb *cheY* gene fragment used as a positive control (lane 4 Fig 4.18a). In both double mutants tested (CAYM6 and CAYM8) a fragment of 10 kb hybridised to the *cheA* probe, in contrast a fragment of approximately 9.2 kb from the wild type N6 hybridised to the same probe. This difference of 0.8 kb represents the presence of the chloramphenicol cassette in the double mutants, CAYM6 and CAYM8. The *cheA* probe also hybridised to the 0.8 kb *cheA* gene fragment used as a positive control (lane 4 Fig 4.18b).

Figure 4.17

PCR analysis of double mutant, CAYM8. Genomic DNA was analysed by specific *cheY*1 primers SPCY3 & SPCY4 and specific *cheA* primers SPCA1 & SPCA2. Lane 1, 1 kb molecular weight marker; lane 2, N6 (SPCY3 & 4); lane 3, N6 (SPCA1 & 2); lane 4, CAYM8 (SPCY3 & 4); lane 5, CAYM8 (SPCA1 & 2); lane 6, pCYIPk1 (SPCY3 & 4); lane 7, pCAIPc1(SPCA1 & 2); lane 8, negative control (SPCY3 & 4); lane 9, negative control (SPCA1 & 2). Negative controls contained PCR mix with primers but without template.

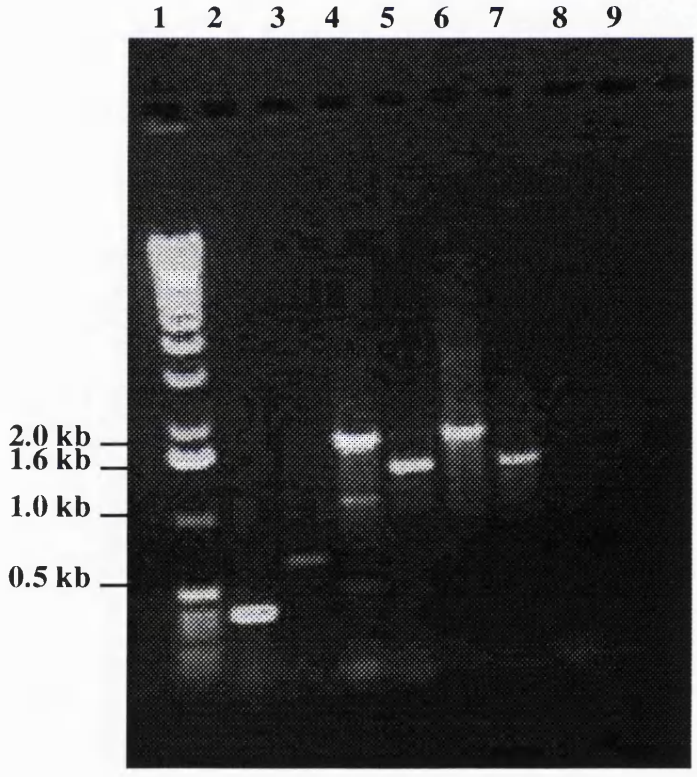
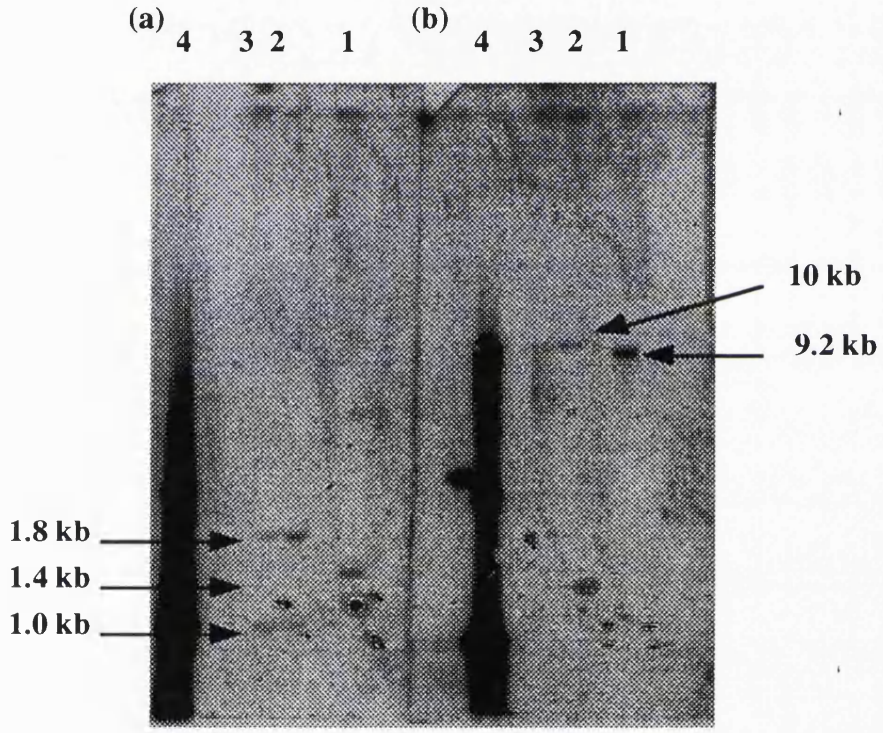


Figure 4.18

Southern hybridisation analysis of *H. pylori* double *cheAY/Y*1 mutants using specific *cheY*1(**a**) and *cheA* (**b**) probes.

(**a**) Lane 1, N6; lane 2, CAYM6; lane 3, CAYM8; lane 4, 1 kb DNA fragment containing *cheY*1, positive control

(**b**) Lane 1, N6; lane 2, CAYM6; lane 3, CAYM8; lane 4, 0.8 kb *cheA* gene fragment, positive control



4.3 Discussion

To investigate the roles of *cheY*1 and *cheAY* in the regulation of motility and chemotaxis in *H. pylori*, defined isogenic mutants were constructed by IPCRM and allelic replacement (Fig. 4.1).

In early studies, undefined *H. pylori* mutants, obtained either by selection for spontaneous variants (Eaton *et al* 1992) or by random chemical mutagenesis (Eaton *et al* 1991), were studied in animal colonisation studies. Since then, precise methods have been developed to construct defined mutants in *H. pylori*, including insertion of an antibiotic resistance cassette into a unique restriction site (Suerbaum *et al* 1993) and random transposon mutagenesis (Ferrero *et al* 1992, Hass *et al* 1993). The method used in this study, inverse PCR mutagenesis (IPCRM), has the advantage of allowing a fragment of the gene to be precisely deleted as well as creating a unique cloning site for insertion of an antibiotic resistance determinant (Wren *et al* 1994, Dorrell *et al* 1996). The selectable markers used in this study both originally from *Camplyobacter coli*; a kanamycin resistance cassette (*aph3'*-III; Labigne-Roussel *et al* 1988) and a gene conferring resistance to chloramphenicol (cat_{GC}; Wang *et al* 1990) are both expressed in *H. pylori*.

Recombinant plasmid DNA can be efficiently transformed into *H. pylori* cells by natural transformation (Nedenskov-Sørensen *et al* 1990) and electroporation (Ferrero *et al* 1992). Several *H. pylori* genes, including *flaA*, *flgE*, *hpaA* and *clpB*, have been mutated using natural transformation (Hass *et al* 1993, O'Toole *et al* 1994, Jones *et al* 1997 and Allan 1997). Electroporation is reported to be more efficient than natural transformation (M^{c}Gowan *et al* 1997), producing rates of transformation in the range of 1.0 transformants/μg to $2x10^3$ transformants/μg of plasmid DNA, depending on the recipient *H. pylori* strain. Successful delivery of the mutated target genes, *flaA*, *flaB* and *fliI*, into *H. pylori* cells has been achieved

using electroporation (Ferrero *et al* 1992, Suerbaum *et al* 1993 and Jenks *et al* 1997).

As outlined in Chapter 1, the three dimensional structure of CheY from *E. coli* and *S. typhimurium* has been determined (Stock *et al* 1989, Volz and Matsumura 1991). The primers used for IPCRM were designed to delete a central portion of the *H. pylori* CheY1 which include three of the conserved sites, 66, 68 and 69 from the α 3 helices (Volz 1993). These sites are thought to be conserved for the structural integrity of the protein (Volz 1993). This resulted in about 900 bp of *H. pylori* specific DNA either side of the inserted kanamycin resistance gene cassette for homologous recombination between the wild type *cheY1* and the mutated allele. At first, attempts where made to construct a mutant using the plasmid construct, pCYIPk1, in which the orientation of transcription of the kanamycin cassette was the same as that of the *cheY*1. The cassette and orientation where chosen to minimise the risk of polar effects. Since this cassette does not contain a transcription terminator, it permits the expression of genes situated downstream (*cheY*1, heat shock methylase, *hsm*, and the essential cell division gene, *fst*H) and thus avoids or greatly reduces potential polar effects (Beier *et al* 1997). Interestingly repeated attempts to create a mutant via natural transformation using this construct failed, presumably due to polar effects which disrupted the *fst*H gene downstream, rendering the mutation lethal to *H. pylori.* Therefore other *cheY*1 clones containing the kanamycin cassette were analysed using a combination of vector and cassette specific primers to identify one in which the kanamycin cassette is in the reverse orientation of transcription to the *cheY*1. Plasmid pCYIPK2 was identified and transformed into the wild type N6 yielding $1x10^5$ kanamycin resistant transformants per μg of DNA. This result was confirmed by Beier *et al* (1997), who also found that insertion of the kanamycin cassette into *cheY*1 which was in the opposite orientation to operon transcription caused no polar effects.

Mutagenesis of the *cheAY* homologue used IPCRM to delete a 37 bp region in the C-terminal portion of the CheA domain (Stock *et al* 1995). Mutagenesis of *cheAY* demonstrated that only a 707 bp gene fragment (pCA110) without any regulatory sequences is necessary for the IPCRM/allelic replacement approach to successfully mutate *H. pylori* genes. As a result of natural transformation into *H. pylori* N6 cells, kanamycin-resistant transformants were obtained at frequencies of $2x10^4$ per μg of plasmid DNA with the kanamycin resistance gene in the same transcriptional orientation as the *cheAY*. Similarly *cheY* was mutated by a 5 bp deletion introduced by IPCRM. Re-introduction of the mutated allele into the wild type N6 cells was performed by electroporation at a transformation frequency $1x10^5$ per μg of DNA. As observed with the mutation of *cheA*, the introduction of the kanamycin cassette into *cheY* in the same transcriptional orientation did not appear to have any polar effects on genes located downstream. The isolation of viable *H. pylori cheY*1, *cheA* and *cheY* mutants indicates that none of these genes are essential for the viability of the organism.

In order to further elucidate the functions of the chemotaxis components in *H. pylori*, a *cheAY/Y*1 double mutant was constructed. A similar approach, used previously to mutate both the flagellin subunits (Josenhans *et al* 1995), took advantage of both the resistance cassettes, *aph3'*-III and cat_{GC}, that can be expressed in *H. pylori.* Electroporation of plasmid, pCAIPc, into *H. pylori cheY*1 mutants resulted in a low number recombinant cells. This was perhaps due to the strong selective pressure of both kanamycin and chloramphenicol on the growth of the cells. With the exception of the experiment to generate a double *cheAY/Y*1, both natural transformation and electroporation were used with similar efficiency for the generation of defined mutants in *H. pylori* throughout this study. PCR and Southern hybridisation analysis confirmed the replacement of the chemotaxis homologues with the mutated alleles.

This study has demonstrated that the construction of defined mutants in *H. pylori* using the IPCRM technique followed by allelic replacement has many advantages over alternative methods and is proven to be a reliable method. Following the construction of *H. pylori cheY*1, *cheAY*, *cheY* and *cheAY/Y*1 isogenic mutants, the next stage of research concentrated on the analysis of their phenotypic characteristics in relation to motility and chemotaxis.

4.4 Summary

To investigate the role of the chemotaxis homologues, CheY1, CheA and CheY, the *cheY*1 and *cheAY* genes of *H. pylori* N6 and SS1 were mutated by IPCRM and allelic replacement. Additionally a *cheY*1 mutant and a double mutant, *cheAY/Y*1, was constructed in *H. pylori* N6. The genotype of each of the defined mutants was confirmed by both PCR and Southern hybridisation. This study demonstrated the usefulness of the IPCRM approach in the construction of defined isogenic mutants in *H. pylori*.

Chapter 5

In vitro analysis of Chemotaxis mutants

5.1 Introduction

Motility is a vital adaptation for many bacterial pathogens capable of colonising mucosal surfaces. *H. pylori* is extremely motile in a viscous environment (Hazell *et al* 1986), a property important for colonisation and persistence in the piglet model (Eaton *et al* 1992, 1996). Chemotaxis which lends a directional component to microbial motility is important for the localisation of other bacterial pathogens *in vivo*. In *Vibrio cholerae*, the importance of chemotactic movement in association with the intestinal mucosa has been shown both *in vitro* and *in vivo* model systems (Freter *et al* 1981a, 1981b). In *C. jejuni*, chemotaxis has been shown to be required for colonisation in animal models (Takata *et al* 1992, Yao *et al* 1997), and a range of chemoattractants have been identified (Hugadahl *et al* 1988).

H. pylori is found in the gastro-intestinal tract only in association with areas of gastric metaplasia (Blaser *et al* 1992), suggesting a specific tropism for gastric mucus secreting cells. Examination of gastric biopsies by electron microscopy found *H. pylori* scattered throughout the mucus layer, near the luminal surface, midlayer and adjacent to the surface mucus cells (Ogata *et al* 1998). Another study demonstrated that *H. pylori* was found particularly at epithelial intercellular junctions on the surface of gastric epithelial cells (Hazell *et al* 1986). It is possible that chemicals found at high concentrations either in mucus, or at gastric intracellular junctions act as attractants for *H. pylori* (Hazell *et al* 1986). *H. pylori* has been shown to exhibit chemotaxis to gastric mucin, urea and bicarbonate (Turner *et al* 1997, Mizote *et al* 1997, Nakamura *et al* 1998).

In this study the histidine kinase component, CheA, and two response regulator components, CheY and CheY1 were mutated in *H. pylori* N6 and SS1 (see Chapter 4). To determine the roles of these components in the chemotaxis system of *H. pylori*, three different experimental systems: swarm plates, capillary assays

and computerised motility analysis, were used to study the defined chemotaxis mutants. Results and discussion of these experiments are presented in this chapter.

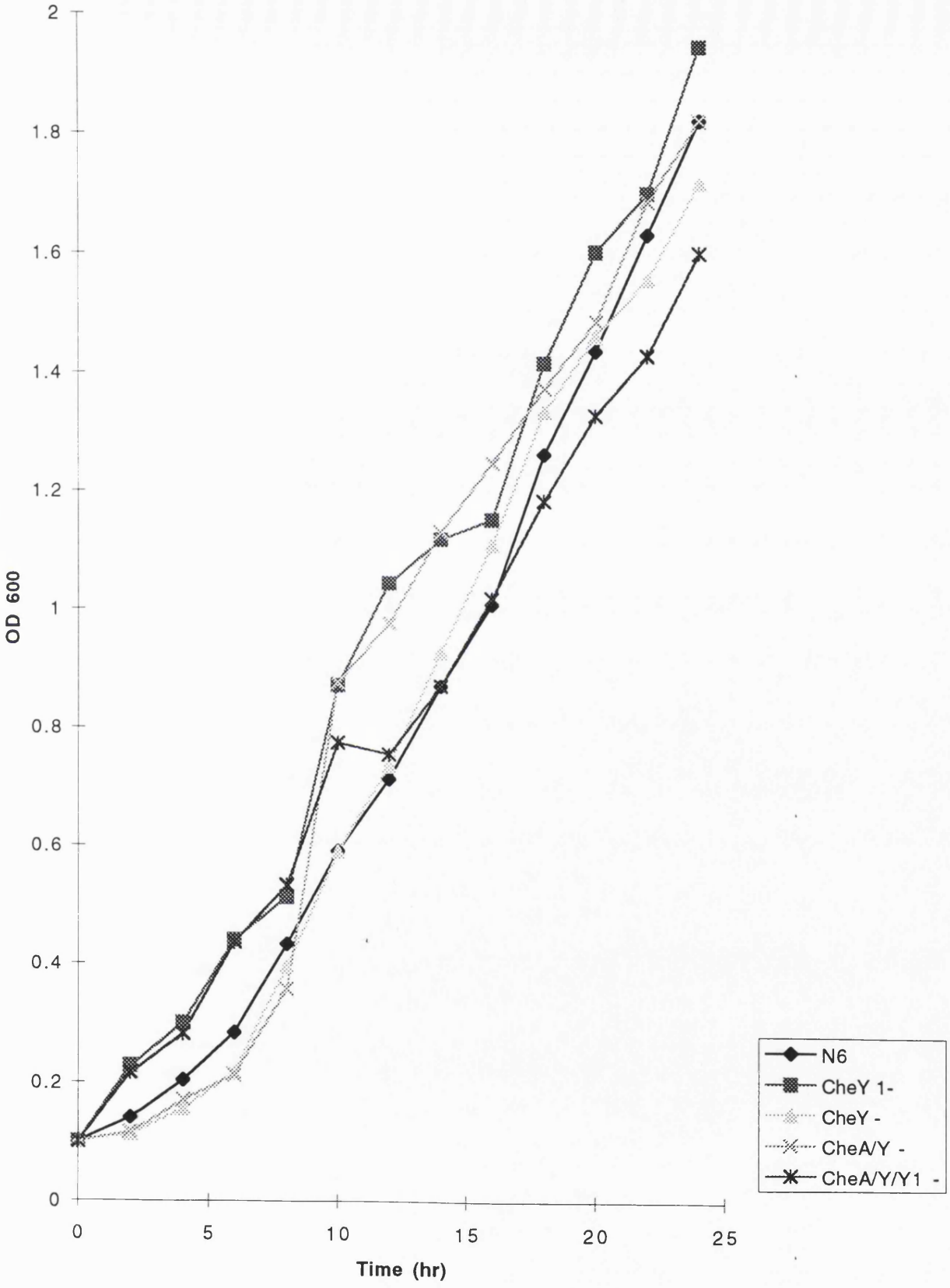
5.2 Results

5.2.1 Analysis of the growth characteristics of the wild type and mutant strains of *H. pylori*

To determine if the mutation has any effect on growth, the growth of the wild type and chemotaxis mutants were compared *in vitro*. This is essential, as any metabolic defect inadvertently caused during the construction of the defined mutants would effect growth of the mutant and subsequent *in vitro* analysis. Growth curves of *H. pylori* N6, N6ΔCheY1$^-$, N6ΔCheY$^-$, N6ΔCheAY$^-$ and N6ΔCheAY/Y1$^-$ were analysed over a period of 24 hours by measurement of optical density (600nm) of cultures inoculated to OD_{600} of 0.1. All experiments were performed in triplicate. The results indicate that there are no differences in growth at 37°C between all strains (Fig 5.1).

Figure 5.1: Comparison of the growth of the wild type and chemotaxis mutants of *H. pylori* N6 *in vitro* by measurement of optical density over 24 hours.

Growth curves of N6 H. pylori strains



5.2.2 Swarm plate analysis

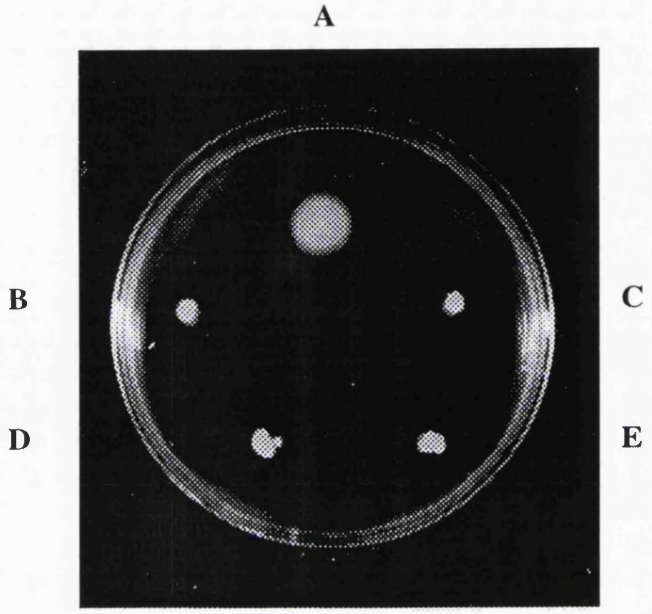
The swarming behaviour of wild type and mutant *H. pylori* N6 and SS1 strains was compared on 0.3% BHI agar plates containing 10% fetal calf serum (Fig 5.2 (a) and (b)). Analysis of these plates showed that the N6ΔCheY1$^-$, SS1ΔCheY1$^-$, N6ΔCheY$^-$, N6ΔCheAY$^-$, SS1ΔCheAY$^-$ and N6ΔCheAY/Y1$^-$ mutants had reduced swarming ability compared to the parent strains. The wild type cells formed concentric rings which increased with the period of incubation. In contrast, the mutants formed irregular growth patterns of a high density limited to the area of inoculation. No rings of growth were observed outside this high density area. The results shown here reflect the limitations and indirect nature of this method.

Figure 5.2: Swarm plates demonstrating the swarming abilties of the wildtype and chemotaxis mutants of *H. pylori* N6 and SS1.

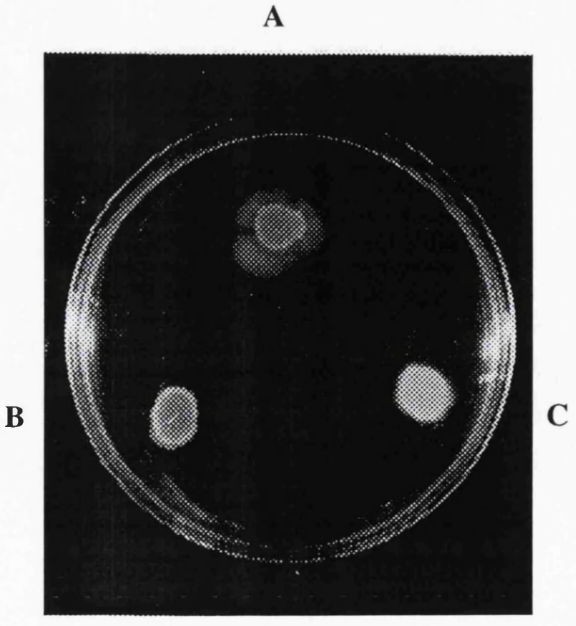
(a) A- N6 wildtype, **B**- N6ΔCheY1$^-$, **C** - N6ΔCheY$^-$, **D** - N6ΔCheAY$^-$, **E** - N6ΔCheAY/Y1$^-$

(b) A- SS1 wildtype, **B**- SS1ΔCheY1 $^-$, **C**- SS1ΔCheAY$^-$

(a)



(b)



5.2.3 Capillary Assays

The ability of the wild type strain N6 and the chemotaxis mutants to respond to hog gastric mucin (HGM) were compared using a modified Adlers method (Adler 1973). Chemotaxis was expressed as a ratio (R_{che}) of the number of bacteria in the mucin capillary tube to the number in the control capillary tube (containing chemotaxis buffer) (Moulton and Montie 1979). An R_{che} of >2 was regarded as significant (Moulton and Montie 1979). *H. pylori* N6 showed significant chemotaxis to 0.1%, 0.5% and 1% wt/vol HGM. Figure 5.3 and Table 5.1 shows the response of N6 to increasing concentrations of HGM. By contrast the N6ΔCheY1$^-$ mutant failed to show significant taxis at 0.1% HGM, but at 0.5% and 1% HGM, chemotactic responses were observed (Table 5.1). These values represent a 90% and 86% reduction in taxis at 0.5% and 1% HGM respectively compared to the wild type strain. Surprisingly, the N6ΔCheAY$^-$ mutant showed a significant chemotactic response to 1% HGM (Table 5.1). However, compared to the wild type strains, taxis of the N6ΔCheAY$^-$ mutant was 94% reduced at 0.5% HGM and 82% reduced at 1% HGM. The N6ΔCheY$^-$ and N6ΔCheAY/Y1$^-$ mutants, did not respond except at the highest concentration of HGM. However the ratios observed were too close to the minimum R_{che} value of 2 to be considered significant (Table 5.1).

Figure 5.3: Response of *H. pylori* N6 and chemotaxis mutant strains to hog gastric mucin analysed by capillary assays. Each point represents a minimum of 3 experiments performed in triplicate. Results are expressed as the ratio of number of bacteria in attractant capillaries to that in control capillary (R_{che}). Standard deviations of the mean are detailed for the N6 strain on the figure and in Table 5.1 for the remaining strains .

Figure 5.3
Response of *H. pylori* strains to Mucin using Capillary assays

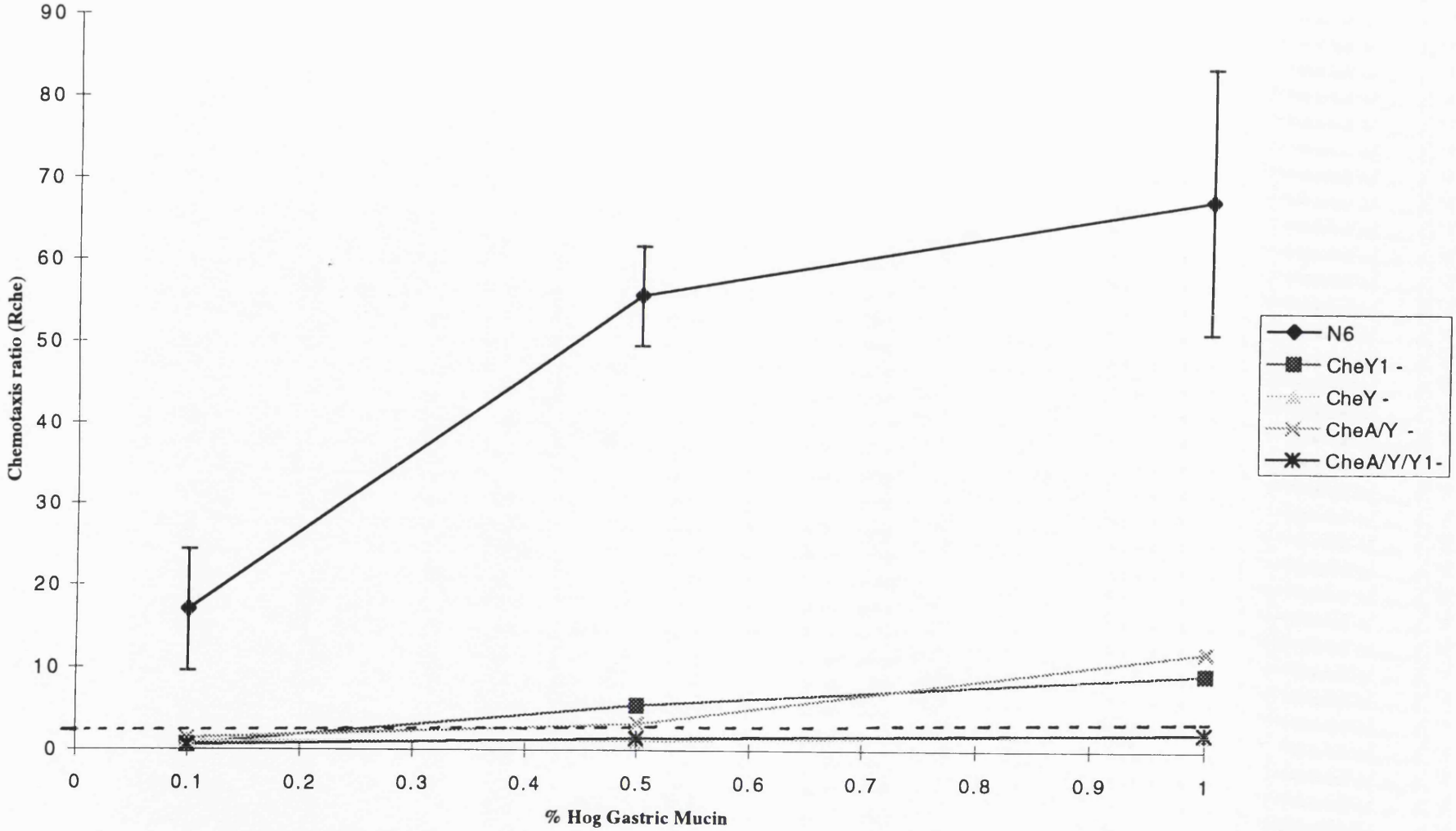


Table 5.1: Response of *H. pylori* N6 and chemotaxis mutant strains to hog gastric mucin analysed by capillary assays. Each data set represents a minimum of 3 experiments performed in triplicate. Results are expressed as the ratio of number of bacteria in attractant capillaries to that in control capillary (R_{che}). NR represents no significant response.

% HGM	0.1%	0.5%	1%
N6	16.96±7.37	55.65±6.08	67.69±16.33
ΔCheY1⁻	NR	5.4±0.84	9.33±1.88
ΔCheY⁻	NR	NR	2.07±0.65
ΔCheAY⁻	NR	3.15±1.74	11.95±5.41
ΔCheAY/Y1⁻	NR	NR	2.08±0.42

5.2.4 Measurement of motility using the Hobson BacTracker system.

The Hobson BacTracker is a motility measuring system which uses a unique "blob and track" imaging processing technology. This system allows bacterial movement to be measured in real time. The tracker comprises a phase contrast microscope connected to a video camera which is linked to a computer. Several parameters of motility including curvilinear velocity, straight line velocity, curvature, stop frequency and stop time frequency can be measured accurately and reproducibly (Karim *et al* 1998). Computer tracking has been used to measure the motility of many bacterial species including *S. typhimurium* (Poole *et al* 1988), *R. spherioides* (Packer and Armitage 1994, Hamblin *et al* 1997) and *R. meliloti* (Sourjik and Schmitt 1996). In this study, real time computer tracking was used to analyse the motility of the N6 wild type strain and chemotaxis mutants in free-swimming conditions and following the addition of urea from at least six seperate cultures.

5.2.4.1 Curvilinear velocity of the wild type and chemotaxis mutants of *H. pylori* N6.

The curvilinear velocity (CLV, μm/s) is defined as the length of a track divided by the time taken to travel it. It is calculated by adding the incremental distances moved in each frame along the sampled path and dividing by the total time for the track.

Three of the deletion mutants, N6ΔCheY$^-$, N6ΔCheAY$^-$, and N6ΔCheAY/Y1$^-$ had significantly higher CLV values than the *H. pylori* N6 wildtype strain. In contrast, N6ΔCheY1$^-$, had a slower swimming speed (Fig. 5.4a, Table 5.2).

5.2.4.2 Straight line velocity of the wild type and chemotaxis mutants of *H. pylori* N6.

The straight line velocity (SLV, μm/s) is the speed of the bacterium in a straight line from the beginning to the end of its path. It is calculated by measuring the straight line distance between the start and end point of the track and dividing by the time taken to travel it.

The SLV of the N6ΔCheY$^-$, N6ΔCheAY$^-$, and N6ΔCheAY/Y1$^-$ mutants were all significantly higher than the wild type strain. In comparison N6ΔCheY1$^-$, showed an SLV lower than that observed for the wild type strain (Fig. 5.4b, Table 5.2).

5.2.4.3 Track linear percentage of the wild type and chemotaxis mutants of *H. pylori* N6.

The Track linear percentage (TL%) is the ratio of the straight line velocity to the curvilinear velocity x 100, (SLV/CLV x 100). Therefore, the more curved the route the bacterium takes, the higher will be the curvilinear velocity, and the lower will be the straight line velocity and the value of the TL%. For a bacterium that swims in an absolute straight line this value may approach 100%.

This parameter indicated that the mutant strains, N6ΔCheY$^-$, N6ΔCheAY$^-$, and N6ΔCheAY/Y1$^-$ are significantly straighter swimming than the wild type (Fig. 5.4c, Table 5.2). The linearity of these mutant strains suggests that both CheY and CheA contribute to the creation of tumbles. In contrast, the linearity of the N6ΔCheY1$^-$ mutant was less than that observed for the wild-type strain.

Table 5.2: The curvilinear velocity (CLV, μm/s), straight line velocity (SLV, μm/s) and the track linear percentage (TL%), as determined by Hobson BacTracker analysis [a].

Strains	CLV μm/s	SLV μm/s	TL%
N6	19.20±0.45	4.91±0.163	25.41±0.889
ΔCheY1$^-$	14.5±2.14	3.51±0.57	24.38±3.70
ΔCheY$^-$	25.56±2.69	10.70±1.89	41.64±3.76
	p=0.0003	*p=0.0008*	*p=<0.0001*
ΔCheAY$^-$	24.21±0.919	13.78±2.02	56.76±1.7
	p=6.4391E-4	*p=1.01031E-8*	*p=1.57712E-8*
ΔCheAY/Y1$^-$	28.38±1.87	17.26±1.76	60.05±3.32
	p=0.00106	*p=4.90497E*	*p=1.4284E-6*

a. Mean values of 100 individual cell tracks were determined from each sample using computerised motion analysis and were averaged from at least five independent cell populations.

b. Statistical significance was determined by the independent t-test.

5.2.4.4 Trail draws

The track linear percentage value allows us to calculate the straightness or curvature of the path of the bacterium. This can also be demonstrated visually using the trail draw facility on the Hobson BacTracker. Trail Draws are diagrams

of the computer tracks of the bacteria which illustrate the different patterns of motility.

Wildtype *H. pylori* N6 moved in a random darting fashion, with frequent change in direction and short straight runs. The chemotaxis mutants, N6ΔCheY$^-$, N6ΔCheAY$^-$, and N6ΔCheAY/Y1$^-$, all moved in long straight runs or very wide circles with no sharp turns or changes in direction. In contrast, N6ΔCheY1$^-$, tumbled excessively, rarely moving out of the field of vision (Fig. 5.5a-e).

Figure 5.4 : Bar charts representing data obtained from the Hobson BacTracker. Error bars show standard deviation.

(a) Bar chart showing the curvilinear velocity (CLV) of *H. pylori* N6 and chemotaxis mutants (μm/s).

(b) Bar chart showing the straight line velocity (SLV) of *H. pylori* N6 and chemotaxis mutants (μm/s).

(c) Bar chart showing the track linear percentage (TL%) of *H. pylori* N6 and chemotaxis mutants.

Figure 5.5 (a-e): Trail draws (diagrams showing paths of individual bacteria tracked by the computer) for the (a) wild-type, (b) ΔCheY1$^-$, (c) ΔCheAY$^-$, (d) ΔCheY$^-$ and (e) ΔCheAY/Y1$^-$ *H. pylori* N6 strains.

Figure 5.4 (a)
Curvilinear velocity of *H. pylori* strains

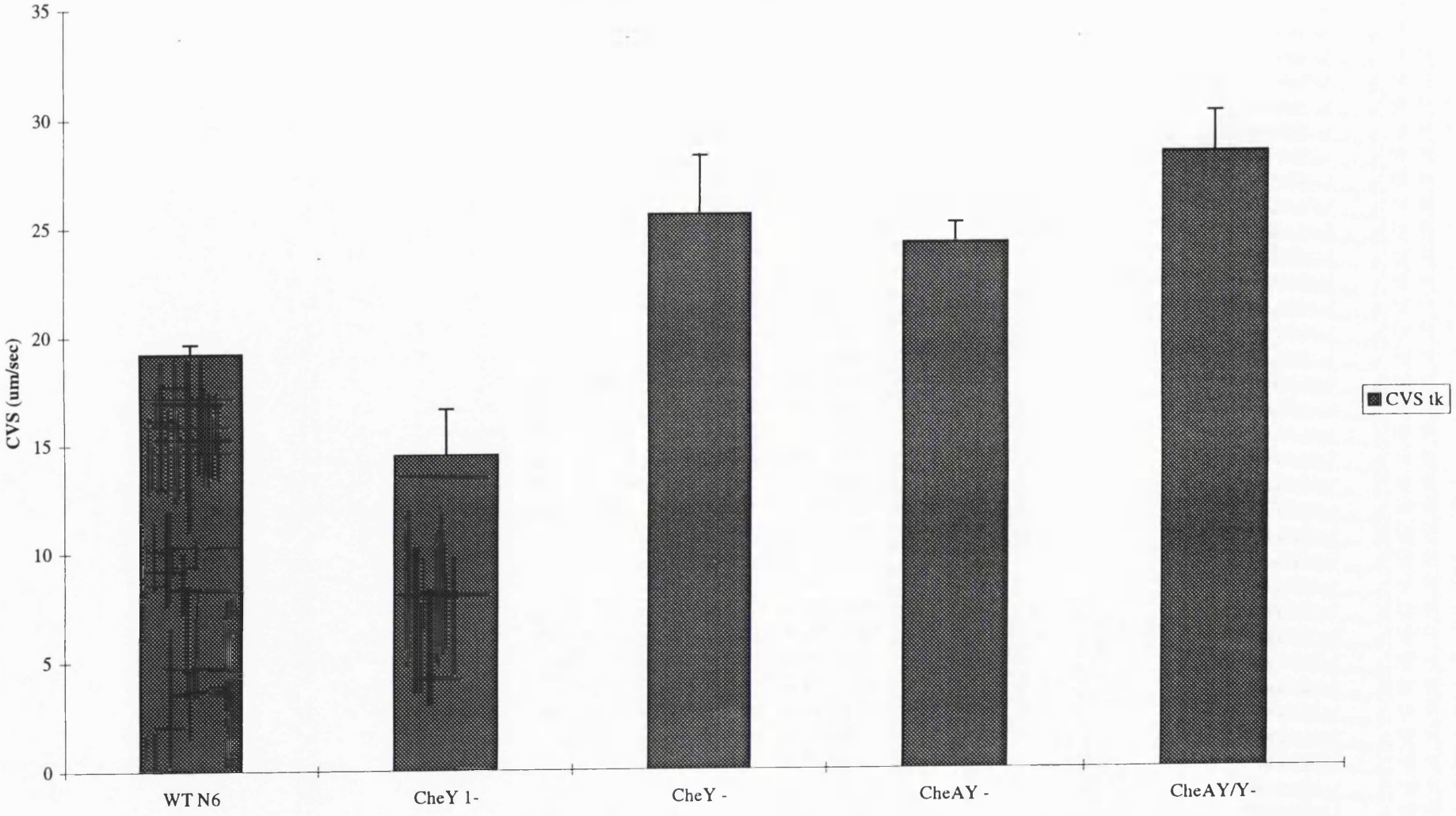


Figure 5.4 (b)
Straight line velocity of *H. pylori* strains

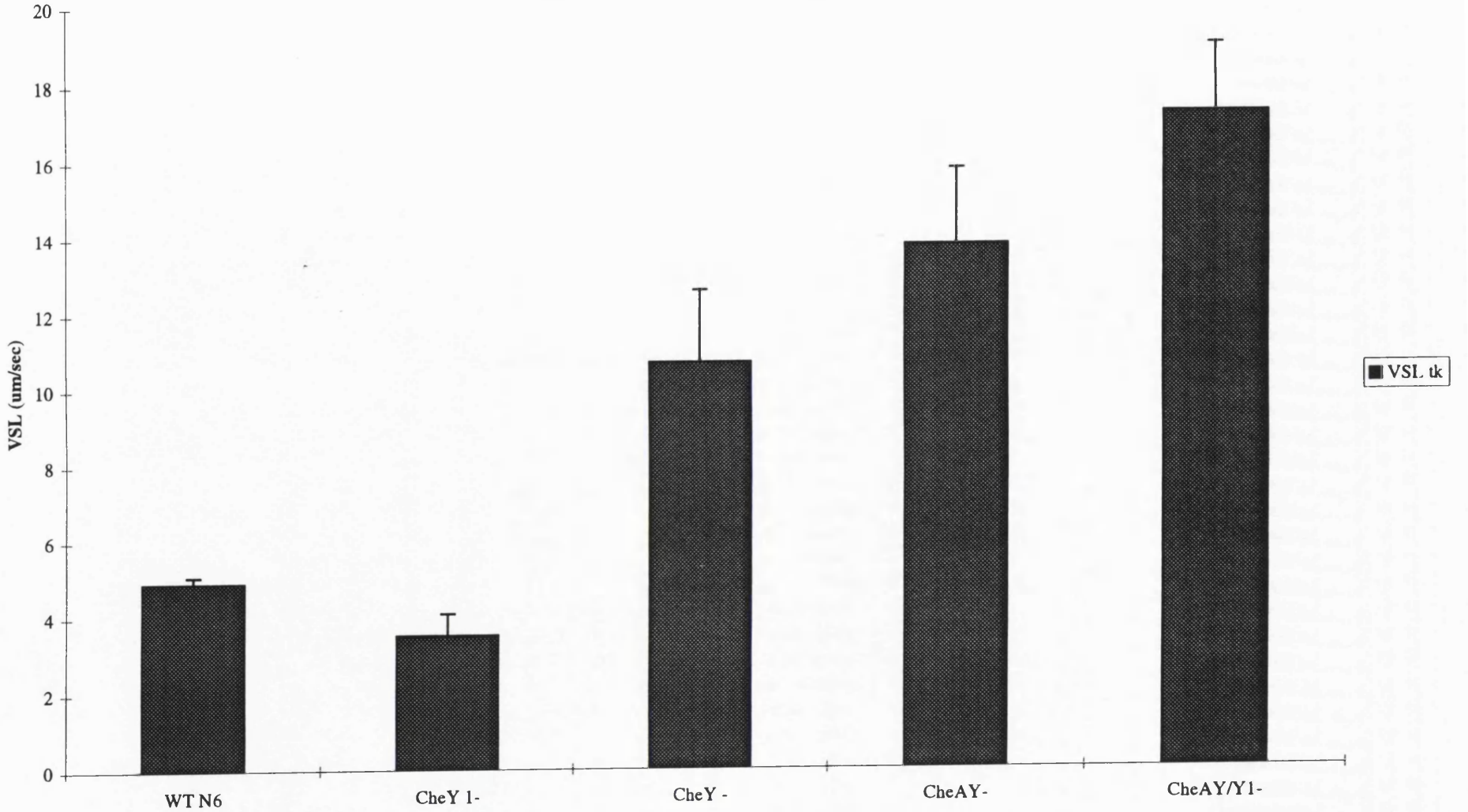


Figure 5.4 (c)
Track linear percentage of *H. pylori* strains

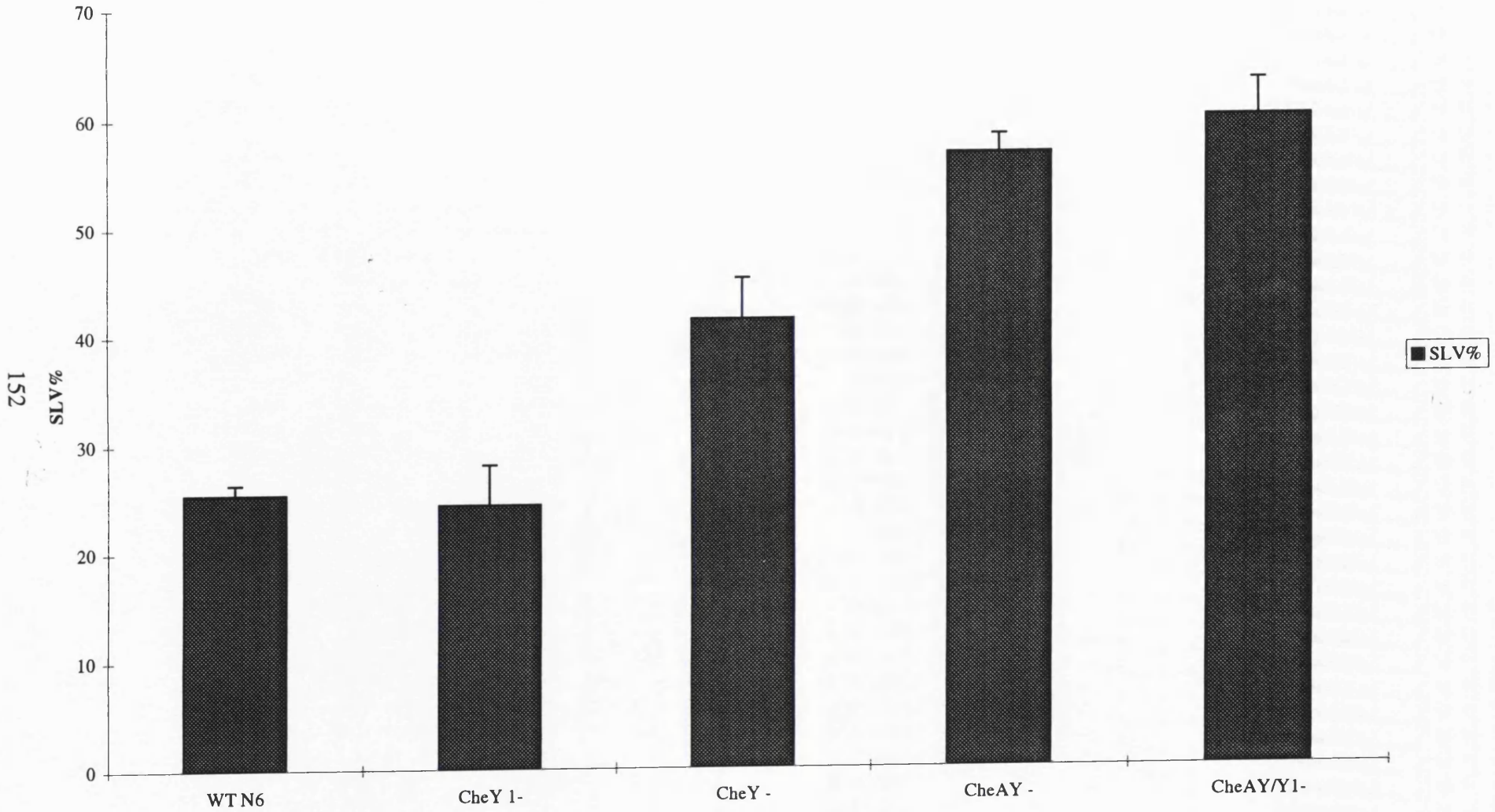
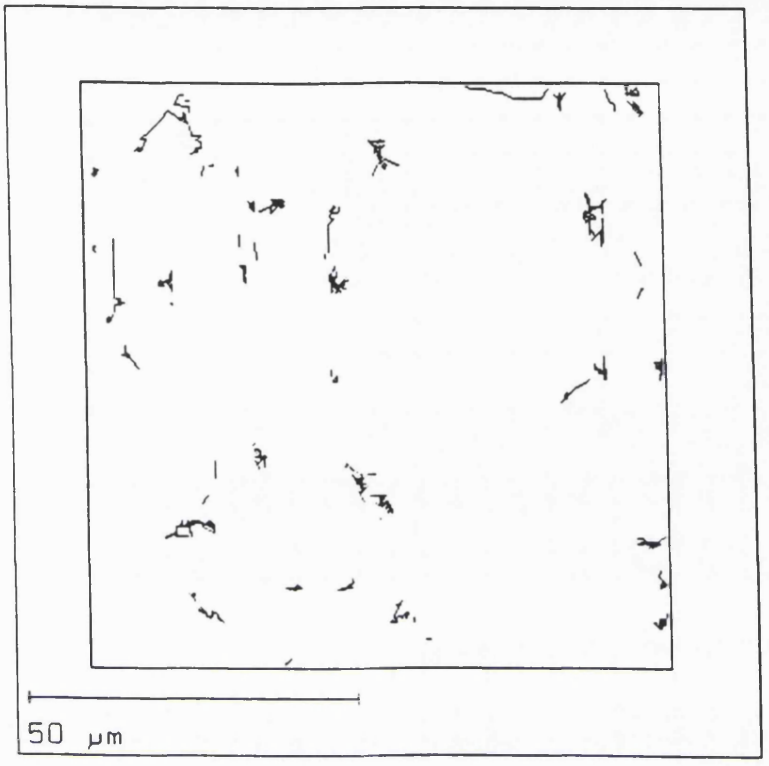
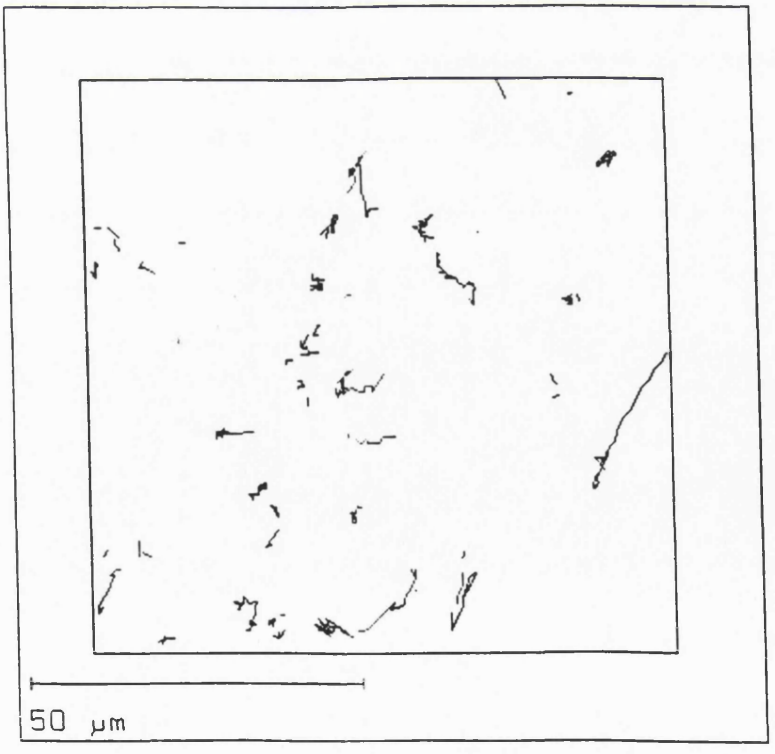


Figure 5.5 (a-e)

(a) N6

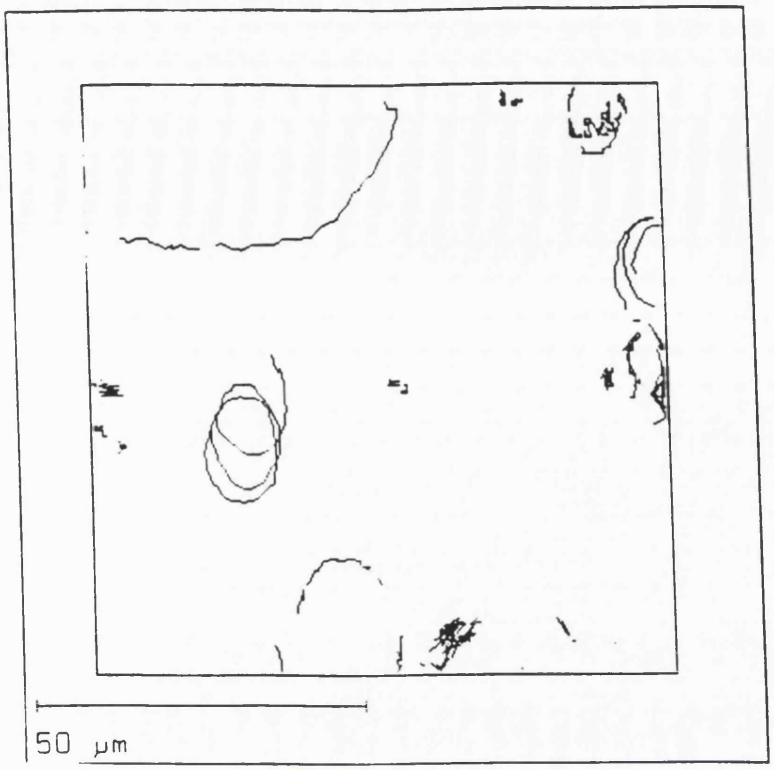


(b) ΔCheY-1



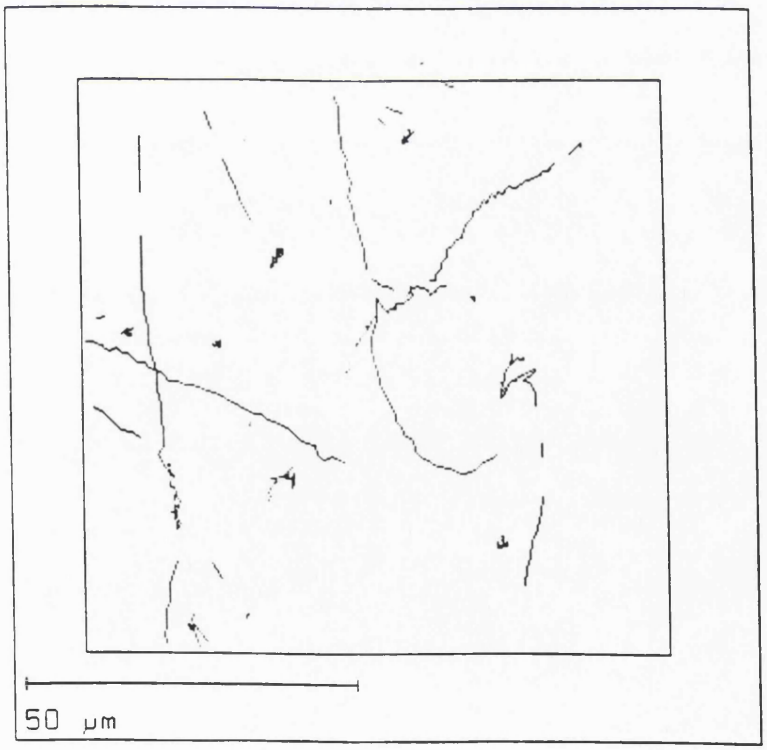
(c)

ΔCheA/Y

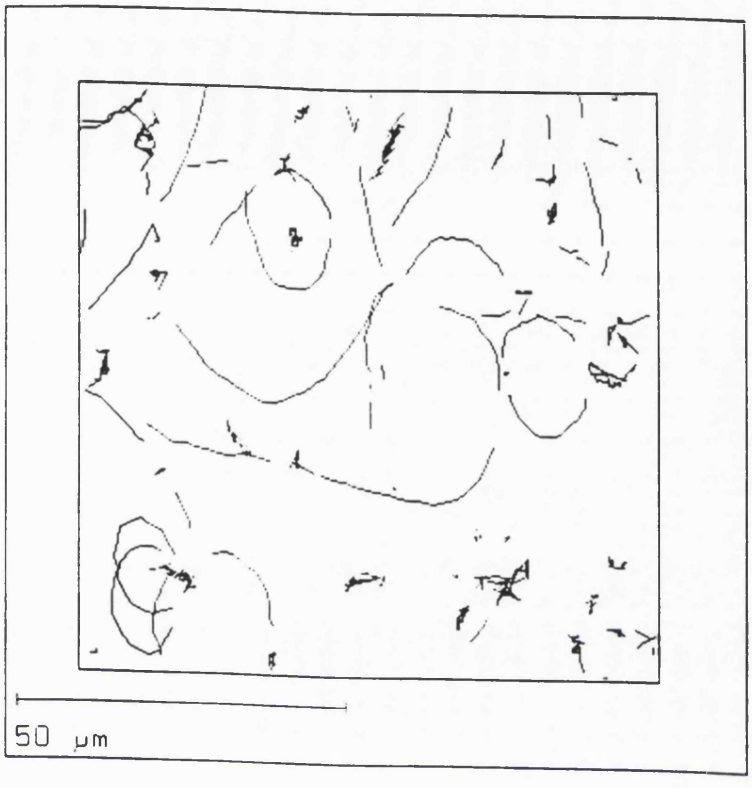


(d)

ΔCheY



(e) ΔCheAY/Y-1



5.2.4.5 Analysis of the swimming behaviour of *H. pylori* strains in the presence of urea.

The tracks of free swimming cells were analysed immediately after the addition of different concentrations of urea. A stepwise increase in urea concentration did not cause an increase in the CLV or swimming speed of wildtype cells. However cells analysed after the addition of 10 mM urea exhibited a 9% increase in smooth swimming rates. This effect, although not statistically significant (p=0.07237), demonstrates the suppression of tumbles in response to a positive stimulus. The N6ΔCheAY$^-$ strain showed a 10% increase in CLV following the addition of 10mM urea. This response to tactic stimulation by increased swimming velocity has been termed "chemokinesis". Concurrent with this response, N6ΔCheAY$^-$ cells upon stimulation by 10 mM urea exhibited a 12% increase in the TL%. Addition of 1 mM or 100 mM concentrations of urea caused no significant deflection in CLV or TL% rates. These results suggest that the mutant, N6ΔCheAY$^-$, still has control of its tumbling ability. The double deletion mutant, N6ΔCheAY/Y1$^-$, is 'locked' in maximum speed with maximum smooth swimming rates. However, following the addition of urea, both the CLV and the TL% values decreased. Addition of 10 mM urea resulted in a 33% decrease in the swimming speed and a 9% decrease in the smooth swimming rates of the double mutant. Similarly, the addition of increasing amounts of urea to N6ΔCheY$^-$ caused a significant decrease in both the CLV and the TL% (Table 5.3, Figure 5.6a&b). Addition of urea to N6ΔCheY1$^-$ caused no alterations from the CLV or TL% values observed for these cells without stimulation (data not shown).

Table 5.3

The effect of urea on the curvilinear velocity and track linear percentage of free-swimming *H. pylori* N6 wild type bacteria and chemotaxis mutants.[a]

	H. pylori strains			
Addition [b]	N6	N6ΔCheY$^-$	N6ΔCheAY$^-$	N6ΔCheAY/Y1$^-$
Curvilinear Velocity (μm/s)				
None	19.20±0.45	25.56±2.69	24.21±0.91	28.38±1.87
Urea (10^{-3}M)	17.42±0.69	22.64±2.08	25.82±1.5	23.52±4.46
	[c]*p=0.07011*	*p=0.0617*	*p=0.38214*	*p=0.050*
Urea (10^{-2}M)	19.32±1.04	20.09±2.71	27±1.59	18.91±2.03
	p=0.92638	*p=0.0240*	*p=0.17542*	*p=0.002*
Urea (10^{-1}M)	18.05±0.49	19.58±2.87	22.4±0.87	20.68±1.10
	p=0.11641	*p=0.0177*	*p=0.19519*	*p=0.0001*
Track Linear %[c]				
None	25.41±0.88	41.64±3.76	56.76±1.7	60.05±3.32
Urea (10^{-3}M)	26.08±0.88	40.82±1.75	52.48±1.7	53.31±5.43
	p=0.6491	*p=0.682*	*p=0.10619*	*p=0.0391*
Urea (10^{-2}M)	27.79±0.80	27.89±3.14	64.82±1.4	54.71±4.73
	p=0.07237	*p=0.0010*	*p=0.00359*	*p=0.060*
Urea (10^{-1}M)	24.97±1.93	23.16±1.99	56.18±3.17	57.24±4.88
	p=0.84009	*p=0.0001*	*p=0.87029*	*p=0.3181*

a. Mean values of 100 individual cell tracks were determined from each sample using computerised motion analysis and were averaged from at least five independent cell populations.

b. The effects of urea were determined within 20 s of its addition to all samples

c. Statistical significance was determined by the independent t-test.

Figure 5.6 Bar charts representing data obtained from the Hobson BacTracker after the addition of urea. Error bars show standard deviation.

(a) Bar chart showing the curvilinear velocity (CLV, μm/s) of the wild-type *H. pylori* N6 and chemotaxis mutants to urea.

(b) Bar chart showing the Track linear percentage (TL%) of the wild-type *H. pylori* N6 and chemotaxis mutants to urea.

Figure 5.6 (a)
Curvilinear velocity of *H. pylori* strains after the addition of urea

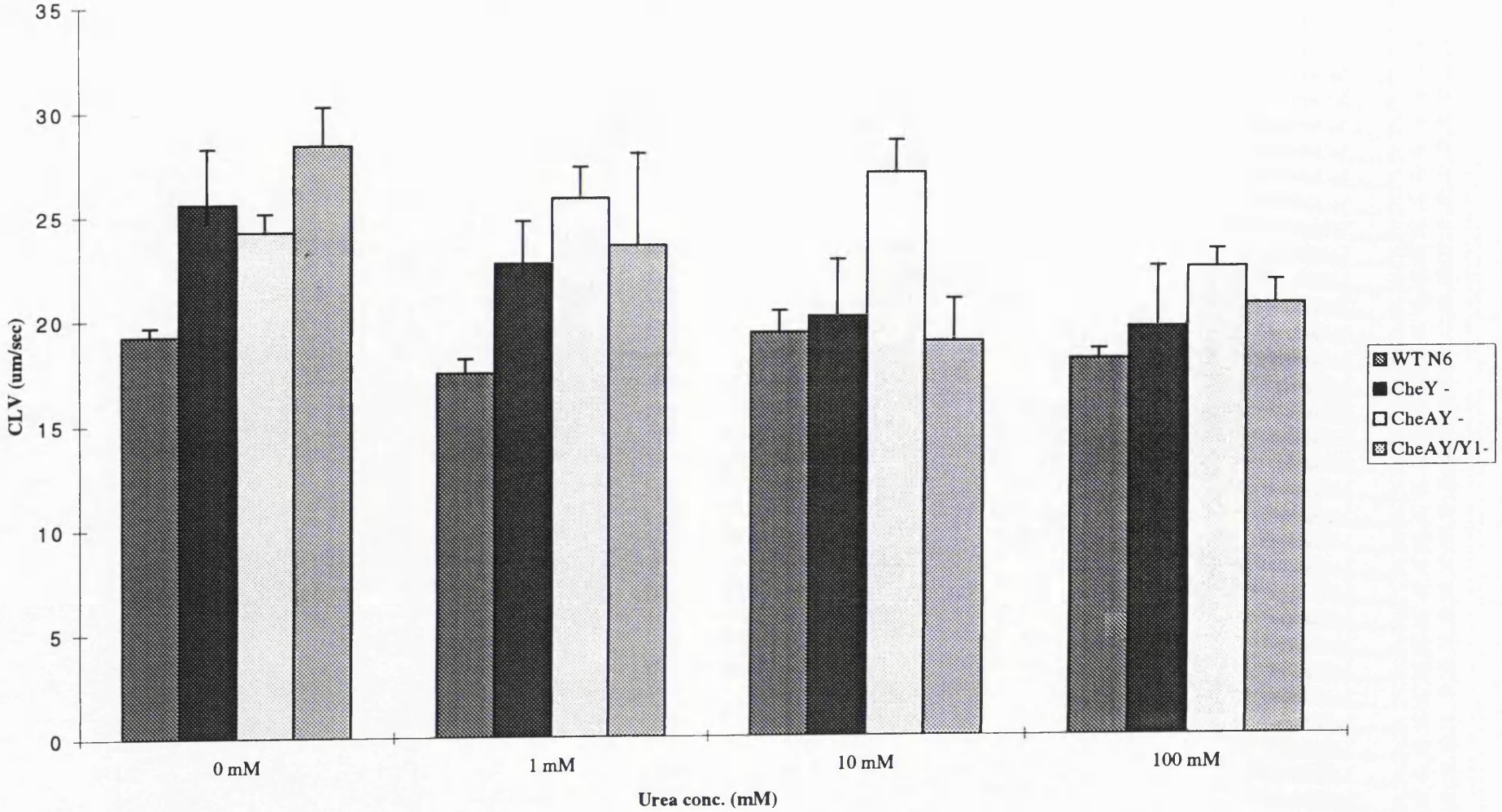
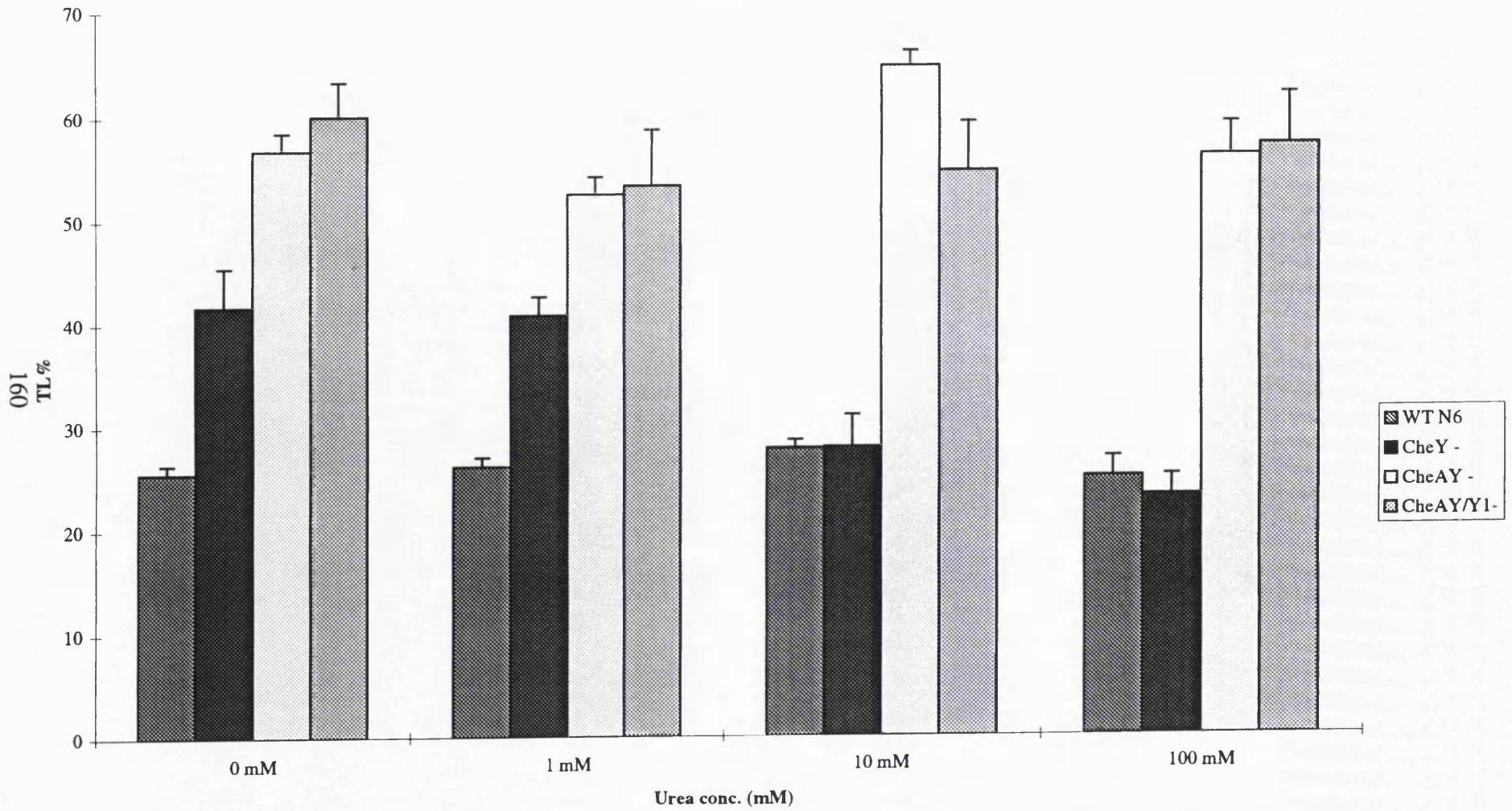


Figure 5.6 (b)
Track linear percentage of *H. pylori* strains after the addition of urea



5.3 Discussion.

To investigate the role of the chemotaxis homologues CheY1, CheA and CheY in *H. pylori*, defined isogenic mutants, constructed by IPCRM and allelic replacement (see Chapter 4), were compared with the parent strains N6 and SS1 using a range of chemotaxis experimental systems.

The validity of data obtained from the chemotaxis assays depends on the assumption that the parent and mutant strains differ only with respect to chemotaxis. Comparison of growth profiles revealed that there were no differences in the growth rate between deletion mutants and the wildtype strain. This suggests that none of the demonstrated phenotypes were the result of metabolic defects caused by the polar effects of the defined mutated genes.

When chemotactic cells of *E. coli* are inoculated onto semisolid nutrient agar they swim outwards in concentric bands. The bands form because of chemotactic responses to spatial gradients generated by transport and metabolism (Wolfe *et al* 1988). Swarm plates have been used in a number of *H. pylori* studies to distinguish mutants defective in flagellar function or production (Suerbaum *et al* 1993, Jenks *et al* 1997). The ability of the chemotaxis deletion mutants to swarm on 0.3% agar plates was greatly reduced compared to that of the N6 and SS1 parental strains. Examination of wildtype and mutant *E. coli* cells in semisolid agar revealed that individual cells swimming in agar behaved as if they were negotiating a 3-dimensional maze (Wolfe *et al* 1988). Microscopic analysis of the wildtype cells showed that the cells migrated through the agar in a series of runs, stops and tumbles. In contrast *E. coli* "null" strains, in which all of the transducers and cytoplasmic chemotaxis proteins were deleted, swam smoothly (Wolfe *et al* 1988). These smooth swimming strains invariably collided with the agar walls, as no tumbles were observed these cells were unable to reorientate themselves efficiently and made little net progress through the agar (Wolfe *et al* 1988). Swarm plates, while convenient and easy to perform, are indirect. Swarming is a

result of numerous factors, including CheY activation via phosphorylation and the ability of activated CheY to interact with and have an effect on the switch. Other influencing factors include growth rate and the metabolic state of the culture. It has been demonstrated in *E. coli* and *S. typhimurium* that some Che$^-$ strains are able to swarm and that swarm size is affected by the intrinsic CW-CCW rotational bias of the switch (Wolfe *et al* 1988).

A modified Adlers capillary assay (Adler 1973), a standard for quantitative assessment of chemotactic proficiency in enteric bacteria, was carried out to study in greater detail the phenotype of the chemotaxis deletion mutants. It has been previously shown that this method of determining the number of cells that accumulate in an attractant-containing capillary is also applicable to *H. pylori* (Mizote *et al* 1997, Turner *et al* 1997). Turner *et al* (1997) using this method, demonstrated that *H. pylori* N6 had a strong positive chemotactic response to hog gastric mucin (HGM). The small, but significant, chemotactic response observed for the N6ΔCheY1$^-$ mutant reveals the importance of a fully functional CheY1 for the full chemotactic response of *H. pylori* to HGM. A similar response was seen by the N6ΔCheAY$^-$ mutant. This response, in the absence of a functional CheAY, suggests that the CheY1 may be phosphorylated by an alternative pathway in response to medium and high levels of mucin. These results demonstrate that mucin is a chemoattractant for *H. pylori* N6, and that the chemotaxis components, CheY1 and CheAY are possibly involved in the control of movement towards the mucus in the stomach. Mucin, a glycoprotein of high molecular weight, is the principle constituent of mucus and is secreted from the epithelial cells of intestinal, gastric and gall bladder tissues. It has been proposed that the L-fucose moiety is the principle chemoattractant in mucin for *C. jejuni* (Hughdal *et al* 1988). Studies have shown that chemotaxis to HGM is resistant to boiling and proteolysis of HGM. Purification and sequencing analysis of the resistant component identified it as a trefoil peptide (R. Logan personal communication). A

trefoil peptide is a conserved epithelial associated molecule which is synthesised along with mucin glycoproteins by specialised epithelial cells and particularly expressed in the antrum of the stomach and at sites of mucosal alteration in the duodenum (R. Logan personal communication). Therefore it may be that trefoil peptides are responsible for attracting *H. pylori* into the gastric mucus overlying the gastric epithelium. This may account for the predominant gastric antral localisation of *H. pylori* infection where trefoil peptides are most abundant (Turner *et al* 1997).

By light microscopy it was possible to demonstrate that the wildtype and all of the chemotaxis deletion mutants were motile. However, light microscopy cannot easily be quantified. For detailed analysis of the movement of *H. pylori* cells, free swimming behaviour under normal conditions and after the addition of the chemoattractant, urea, Hobson Bac Tracker analysis was performed. A difference in the swimming speed (CLV) was observed and quantified for the deletion mutants, ΔCheY$^-$, ΔCheAY$^-$ and ΔCheAY/Y1$^-$, which are significantly faster than the parental strain N6, which is faster than the deletion mutant, ΔCheY1. The straight line velocity (SLV) also showed the same ranking. The smooth swimming value (TL%), defined as the ratio of SLV to the CLV, demonstrated that the wild type strain N6 had a TL% similar to that previously reported for *H. pylori* (Karim *et al* 1998). Strain ΔCheY$^-$ was significantly straighter swimming than the wild type. In *E. coli*, when phosphorylated CheY interacts with the flagellar motor, the consequence of this binding is clockwise rotation or tumbling. Conversely in a mutant lacking CheY, counter clockwise rotation of the flagella results in straight line swimming (Stock and Surette 1996). The straight swimming phenotype observed for ΔCheY$^-$ suggests that the chemotaxis system may be mechanistically similar to that of *E. coli*. The observation that increasing the number of deleted chemotaxis components resulted in straighter swimming strains suggests that CheY1, CheY and CheA all play a role in the generation of tumbles in *H. pylori*

N6. However, examination of the motility of ΔCheY1$^-$, which had a CLV, SLV and TL% lower than that observed for the wild type, suggests that the role played by CheY1 in the generation of tumbles under normal conditions is not significant. It is possible that the slow swimming phenotype of ΔCheY1$^-$ may be due to polar effects resulting from the mutation. However, as summarised in Chapter 4, studies by others have demonstrated that both *copA* and *ftsh*, located downstream of CheY1, are fully functional in a ΔCheY1$^-$ mutant (Beier *et al* 1997). The ratio of SLV to CLV of the ΔCheY1$^-$ mutant indicate motility behaviour closer to that of the wild type with respect to the frequency of directional changes. These results could be possibly explained by studies in *S. typhimurium* involving mutations in the flagellar switch which suppress mutations in CheY1 (Sockett *et al* 1992). A ΔCheY1$^-$ suppression mutation would have resulted in a CW bias, so that the overall switch bias in the suppressed ΔCheY1$^-$ mutant was brought closer to the wild type. This in turn would have resulted in the restoration of the ΔCheY1$^-$ cells ability to swarm. However, swarming was not observed for the ΔCheY1$^-$ strain. The chemotaxis and motility systems of bacterial species are known to be complex, and the extent to which genetics can clearly and reliably determine protein interactions depends on the phenotypic assay and on the number of interacting components. It may be that the slow swimming motility of the ΔCheY1$^-$ strain is due to altered protein interactions or conformational changes which have affected the motility but not the chemotaxis of the strain, thus resulting in altered Hobson BacTracker results which are due to alterations in motility but not in chemotaxis.

The phenotypes of the chemotaxis mutants were further studied in the presence of urea. The increase in the smooth swimming rate of *H. pylori* N6 following the addition of 10 mM urea reflects the suppression of tumbles in response to a chemoattractant. This result is consistent with the studies of Mizote *et al* (1997) which demonstrated that 10 mM urea was a chemoattractant for *H. pylori* in

capillary assays. The positive response of ΔCheAY$^-$ to 10 mM urea suggests that the mutated cells can respond either via CheY1 or by an alternative pathway. This result is consistent with the significant response observed by the ΔCheAY$^-$ mutant to high levels of mucin in the capillary assay. The current state of knowledge about chemotaxis is insufficient to account for the above experimental observations, however there are a number of experimental results which imply that other sites and processes in addition to CheY phosphorylation may be involved in the control of bacterial chemotaxis. In other bacterial systems there is evidence that chemotaxis to dominant attractants (in the case of *H. pylori*, urea and hog gastric mucin) require transport into the cell and, at least limited, metabolism. This proposed system for *H. pylori* is further supported by the finding of a soluble MCP like homologue in *H. pylori* (Tomb *et al* 1997, Allan 1997). Therefore in order to be sensed, chemoattractants could be transported into the cell. Soluble Tlps (transducer-like proteins) have been reported in *R. sphaeroidis*, where it has been demonstrated that attractants must be transported into the cell, and in the case of alanine, subsequent metabolism is required (Poole *et al* 1993). The mechanism which allows this bacterium to respond to repellents, which are presumably not transported into the cell, remains to be elucidated (Poole *et al* 1993). It is possible that in *H. pylori* phosphorylation is controlled through metabolism, either via one of the alternative histidine protein kinases identified by homology (Tomb *et al* 1997) or by direct phosphorylation of CheY1 or one of the CheY domains of CheV by a small phosphodonor linked to general metabolic activity. There is evidence that enteric CheY can be phosphorylated *in vitro* by acetyl phosphate (Mc Cleary 1994) and that metabolites such as fumarate can alter CheY activity (Barak *et al* 1995)

Both deletion mutants, ΔCheY1$^-$ and ΔCheAY/Y1$^-$, appear to be repelled (decrease in swimming speed, increased tumbling) by the addition of urea. This response to urea by ΔCheAY/Y1$^-$ demonstrates that the bacterium is still capable

of chemotaxis. Why *H. pylori* requires and exhibits negative chemotaxis to urea in the absence of the three chemotaxis components (CheY1, CheAY) is unknown. The aerotaxis system of *E. coli* contains two transducers which respond to oxygen, Aer, which mediates positive aerotaxis and Tsr, which in the absence of Aer, mediates a negative response (Manson *et al* 1998). A similar system is possible in *H. pylori* where, in the absence of the CheY1 and CheAY proteins, one of the putative MCP's mediates a repellent response to urea via an alternative histidine protein kinase and response regulator resulting in the increased CW rotation of the flagellae and tumbling of the cells. A similar response to urea was also identified in the ΔCheY$^-$ deletion mutant. This result cannot be explained by the mechanism proposed above. As discussed in Chapter 3, a CheZ homologue was not identified in the genome sequence of *H. pylori* (Tomb *et al* 1997). The function of CheZ in *E. coli* is to facilitate the dephosphorylation of CheY-P (Hess *et al* 1988). It has been proposed for *R. centenum*, which also contains two CheY homologues, that CheY1, which was demonstrated by capillary assays to be essential for the full tactic response, acts as a competitor of CheY2 for phosphorylation by CheA in the absence of CheZ. Similarly in *H. pylori* this study showed that CheY1 was required for the full tactic response to both HGM and urea. Additionally, the ΔCheY1$^-$ mutant resulted in a tumbly phenotype, similar to that observed for a ΔCheZ$^-$ mutant in *E. coli* (Hess *et al* 1988). This suggests a role for CheY1 as a CheZ-like competitor, while the CheY acts as the regulator, interacting with the switch. This proposed scheme would explain the tumbling response of ΔCheY1$^-$ to the addition of urea been caused by an upset in the balance of the phospho-sink, leading to incorrect phosphorylation of the CheY protein. This proposed system is further supported by identification of a soluble MCP in *H. pylori*, which would allow the formation of a soluble complex between the CheW-CheAY and the receptors whilst allowing communication with the

polar flagella. These results demonstrate that CheY1, CheA and CheY are all involved in chemotaxis in *H. pylori*.

5.4 Summary

The role of the CheY1, CheA and CheY chemotaxis homologues in the chemotaxis system of *H. pylori* was investigated using defined isogenic mutants, ΔCheY1$^-$, ΔCheAY$^-$, ΔCheY$^-$ and ΔCheAY/Y1$^-$ in a range of chemotaxis assays. Swarm plate analysis showed that all of the *H. pylori* N6 and SS1 chemotaxis mutants did not have the ability to swarm. Chemotaxis toward mucin by the deletion mutants was greatly reduced or absent in all of the N6 chemotaxis mutants. Finally, analysis of the chemotaxis mutants under normal conditions and after the addition of urea, using the Hobson BacTracker, revealed the roles played by the CheY1, CheA and CheY in the chemotaxis system of *H. pylori* N6. The results of the three chemotaxis experimental analysis lead to a proposed system of chemotaxis which combine features from that of *E. coli* and the less defined *R. centenum.*

Chapter 6

Further analysis of chemotaxis mutants by SDS-PAGE and colonisation studies in animals.

6.1 Introduction

6.1.1 Analysis by SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE) are currently the most commonly used electrophoretic technique for the analysis of proteins. One-dimensional (1D) PAGE gels have been used extensively in *H. pylori* research (Perez-Perez and Blaser 1987). This method allows visualisation of highly expressed proteins in *H. pylori*, including UreA, UreB, FlaA and FlaB after coomassie blue or silver staining. Analysis of the *H. pylori* 26695 genome sequence suggests a total of 1590 open reading frames (Tomb *et al* 1997). 1D SDS PAGE cannot resolve such a large number of proteins. Another limitation of 1D PAGE analysis is that the proteins are separated only on the basis of size and, as a result, each band will contain all of the proteins from the organism with the same properties (Dunn 1993).

The coupling of isoelectric focusing (IEF) in the first dimension and SDS-PAGE in the second dimension resulted in a two dimensional (2D) method that separates proteins according to two different parameters, charge and size. This method, which theoretically can resolve up to 10,000 proteins, has been extensively used in both eukaryotic and prokaryotic research (Dunn 1993). In theory, on each 2D gel, the entire complement of the proteins expressed by a cells genome or a subcellular location (e.g cell envelope) (the proteome) should be visible. Individual proteins, cut out of a 2D gel can be analysed by MALDI-TOF spectrometry, and the "mass fingerprints" of each protein compared to those predicted for the genome database (Pennington *et al* 1997). There are a number of proteome projects currently ongoing in different micro-organisms, including, *Saccharomyces cerevisiae*, *E. coli* and *Haemophilis influenze* (Pennington *et al* 1997). Proteins initially expressed by a particular organism under standard conditions are identified and their position on 2D gels mapped, to provide a "constitutive" or reference map for the organism. By comparison with the

reference map it is then possible to analyse changes in protein expression that occur in response to changing physiological conditions or assign function to gene products after over-expression or knockout of the gene of interest (Pennington *et al* 1997).

To date, the few studies using 2D gel analysis on *H. pylori* have identified porin and oxidative stress protein homologues (Exner *et al* 1995, McGowan *et al* 1997). There is no reference map available for *H. pylori*, but recently a number of antigenic proteins (UreA, UreB, FlaA, FlaB, HpaA) were identified by 2D gel analysis followed by Western blotting with patient serum and peptide mapping by MALDI-TOF mass spectrometry (McAtee *et al* 1998). Additionally, a number of proteins with unknown function (ORFans) were identified as potential virulence proteins (McAtee *et al* 1998). This study demonstrated the potential usefulness of proteomics combined with the genome database of *H. pylori* 26695 in the identification of the role of unknown ORFans and other proteins in *H. pylori* infection and disease.

6.1.2 Animal studies

The gnotobiotic piglet was the first animal model successfully infected with a human isolate of *H. pylori* (Krakowka *et al* 1987, Lambert *et al* 1987). Infection is established within 24 hr of oral feeding and persists for at least 12 weeks. Chronic gastritis occurs in all piglets and increases in intensity with time. From week 2 onwards, large lymphoid follicles in the submucosa and lamina propria are observed. This is accompanied by the identification of *H. pylori*, mostly in the superficial mucus-secreting area of the gastric epithelium (Akopyanzs *et al* 1995, McColm 1997). Gnotobiotic piglets reproduce many features of disease associated with *H. pylori* in humans, however they cannot be used to study the impact of chronic infection owing to the difficulties in maintaining them within isolators long-term (maximum 45-60 days). An additional drawback of this model

is the characteristic patchy distribution of *H. pylori* over the gastric mucosa (Engstrand *et al* 1992) which may result in false negative results for cultured endoscopic biopsies. A more accurate method is to culture the contents of the stomach (Eaton *et al* 1996) or to carry out precise topographic mapping of urease distribution in the gastric mucosa (Engstrand *et al* 1992). Obviously both of these methods involve the sacrifice of the animal which is very costly. However, the gnotobiotic piglet model has been used extensively to study the function of a number of *H. pylori* virulence determinants including urease (Eaton *et al* 1991, Eaton *et al* 1994) the flagellin genes, *flaA* and *flaB* (Eaton *et al* 1996) and the vacuolating cytotoxin, *vacA* (Eaton *et al* 1997). A number of clinical isolates, primarily N6 and 26695, are used in gnotobiotc piglet studies.

Proof that gastric colonisation with *H. pylori* could occur in mice was first reported by Karita *et al* (1991) who showed that Balb/c nude mice could support long-term colonisation with associated gastritis. More recently a number of improved models have been described, including the specific-pathogen free (SPF) CD1 mice or conventional (CV) Balb/c mice, which show good colonisation and develop gastric pathology resembling human disease after oral challenge with mouse adapted fresh clinical isolates of *H. pylori* (Marchetti *et al* 1995). In addition, a simple mouse model of gastric colonisation using HSD/ICR mice has been developed by a group at GlaxoWellcome, which can be colonised by the majority of fresh clinical isolates. Although no significant gastritis is observed in this model, it has been shown to be a rapid screening method for the identification and initial selection of novel anti-*Helicobacter* agents (M^{c}Colm *et al* 1995, M^{c}Colm 1997).

A clinical isolate, the Sidney strain (SS1), found to colonise and cause chronic active gastritis in the majority of mouse models has been proposed as the standard strain for use in vaccine development, compound screening and studies in pathogenesis (Lee *et al* 1997). Further, *H. pylori* SS1 infection in mice induces

humoral immune responses that closely mimic those observed for human *H. pylori* infection (Ferrero *et al* 1998). This will allow both the study of host immune response during acute and chronic stages of *H. pylori* infection, and further investigation of the role of host factors in resistance to *H. pylori* infection.

In Chapter 6, two approaches were used to study the role of the chemotaxis genes in *H. pylori* infection:

(1) 1D SDS PAGE gel was used to compare the protein profiles of the *H. pylori* N6 and SS1 wildtype and their respective chemotaxis mutant strains. Preliminary 2D SDS gel analysis was carried out on the wildtype *H. pylori* N6 and chemotaxis mutant strains and the resulting gels compared to identify the predicted location of CheAY and CheY1 on the proteome of *H. pylori.*

(2) The ability of *H. pylori* N6 and SS1 wildtype and chemotaxis mutant strains to colonise the gnotobiotic piglet model and the GlaxoWellcome mouse model, respectively, were analysed.

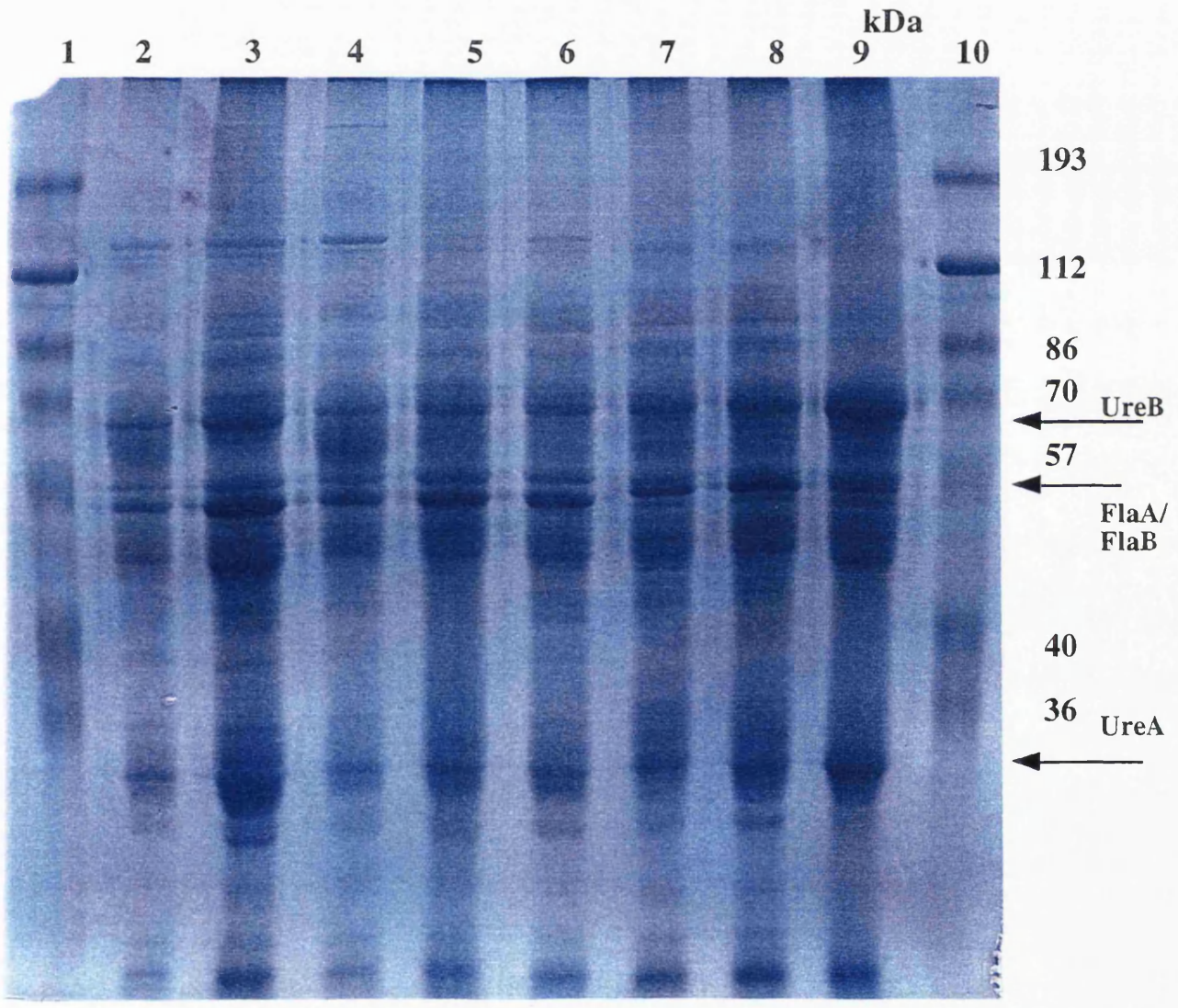
6.2 Results

6.2.1 1 and 2D SDS PAGE gel analysis

6.2.1.1 1D protein gel analysis of the *H. pylori* wildtype strains, N6 and SS1, and their respective chemotaxis mutants.

Total protein from *H. pylori* wildtype and mutant strains were isolated from liquid cultures grown in standard conditions (section 2.1) and an equal amount compared on a 12% 1D SDS PAGE gel (see section 2.6.4.1). There were no obvious differences in the protein profiles between the wildtype *H. pylori* strains N6 and SS1 and their respective chemotaxis mutants (Fig 6.1). This indicates that deletion of the chemotaxis genes, *cheAY* and *cheY1*, has no effect on the protein production of the major proteins, UreA (31 kDa), UreB (66 kDa) and FlaA/FlaB (56/54 kDa) (Fig 6.1).

Figure 6.1 1D protein profiles of *H. pylori* wildtype N6 and SS1 strains and their respective chemotaxis mutants. Lane 1, Protein molecular weight marker, lane 2, N6, lane 3, N6ΔCheY1$^-$, lane 4, N6ΔCheY$^-$, lane 5 N6ΔCheAY$^-$, lane 6, N6ΔCheAY/Y1$^-$, lane 7, SS1, lane 8, SS1ΔCheY1$^-$, lane 9, SS1ΔCheAY$^-$ and lane 10, Protein molecular weight marker.



6.2.1.2 2D SDS PAGE gel analysis

The 2D PAGE profile of a whole cell preparation from the parental *H. pylori* N6 and ΔCheY1-, ΔCheAY$^-$ and ΔCheAY/Y1$^-$ mutated strains are shown in Figures 6.2-6.5. The first dimension of each sample was separated in parallel using an immobilised non-linear gradient of pH 3-10, and the second dimension in a 12-14% SDS gradient gel (see section 2.6.4.2). Isoelectric points (pIs) ranged from approximately 4-8, and the molecular weights ranged from 10-110,000 kDa. It should be noted that both the pI and kDa values quoted for proteins throughout this section were estimated from the genome sequence of *H. pylori* 26695 (Tomb *et al* 1997) using the TagIdent prosite tool on Swiss Port (http://www.expasy.ch./www/guess-prot) and are therefore only estimates for what these values may actually be in *H. pylori* N6. The silver stained gel of the parental *H. pylori* N6 showed numeous individual proteins and several protein families, most notably as clusters of bands (charge trains). These included bands at 78 kDa (pI 4.9), 75 kDa (pI 4.7-5.4), 66 kDa (pI 4.6-5.5) and 33 kDa (pI 5.9-6.9). These clusters and other highly expressed spots are also conserved in all the chemotaxis mutant proteomes and are indicated in all gels by circles (Fig. 6.2-6.5). The pI values indicated on each 2D gel are estimates from 2D SDS PAGE standards run under identical conditions in our laboratory (Dr K. Williams).

6.2.1.2.1 Identification of CheY1 and CheAY 2D PAGE reference points.

Comparitive analysis of the 2D Page was carried out to putatively identify the "protein spots" for CheY1 and CheAY based on their estimates pI and MW. The predicted pI and MW of CheY1 is 5.26 and 13.91 kDa, respectively. On the wildtype *H. pylori* N6 proteome, no proteins spots with these values were found. Additionally, comparison of the N6 wildtype with the ΔCheY1$^-$ and ΔCheAY/Y1$^-$ 2D gels failed to identify any protein of that MW which was present in the wildtype gel but absent in both the ΔCheY1- and ΔCheAY/Y1$^-$

proteomes. The predicted pI and MW for CheAY is 4.98 and 89.75 respectively. One spot absent in both the ΔCheAY$^-$ and ΔCheAY/Y1$^-$ but present in the parental N6 and ΔCheY1$^-$ proteomes has been identified at approximately the correct position and is indicated in all 2D gels (Figs 6.2-6.5). The other major difference between ΔCheAY$^-$, ΔCheAY/Y1$^-$ and the N6 2D gels is the absence of three proteins ranging in size from 12-15 kDa, all of which have the same pI of 6.9-7.0. The area where these proteins are located is indicated by box A in all gels (Fig 6.2-6.5).

6.2.2 Results of animal studies

6.2.2.1 Colonisation of piglets by *H. pylori* N6 wildtype and chemotaxis mutant strain.

The extent of bacterial colonisation was quantified by serial plate dilution on day 7. After colonisation with the wildtype N6 strain, 10^3 and 10^5 CFU/g of gastric mucosa, was recovered in both piglets challenged (Table 6.1). In contrast the *cheY1* deletion strain from three independent bacterial batches failed to colonise all three gnotobiotic piglets challenged (Table 6.1). No altered pathology was observed in either stomach colonised by the *H. pylori* N6 wildtype strain.

Table 6.1 Colonisation of gnotobiotic piglets by *H. pylori* N6 wildtype and *cheY*1 mutated strains.

H. pylori strain	No. infected/total	Challenge inocula	Colonisation CFU/g mucosa
N6	2/2	$10^7/10^8$	$2x10^3/2x10^5$
N6ΔCheY1$^-$	0/3	10^9	0

Figure 6.2: 2D SDS PAGE gel of H. pylori N6 .Circles represent highly conserved proteins. BoxA indicates proteins that may be regulated by CheAY. CheAY is also indicated. No putative

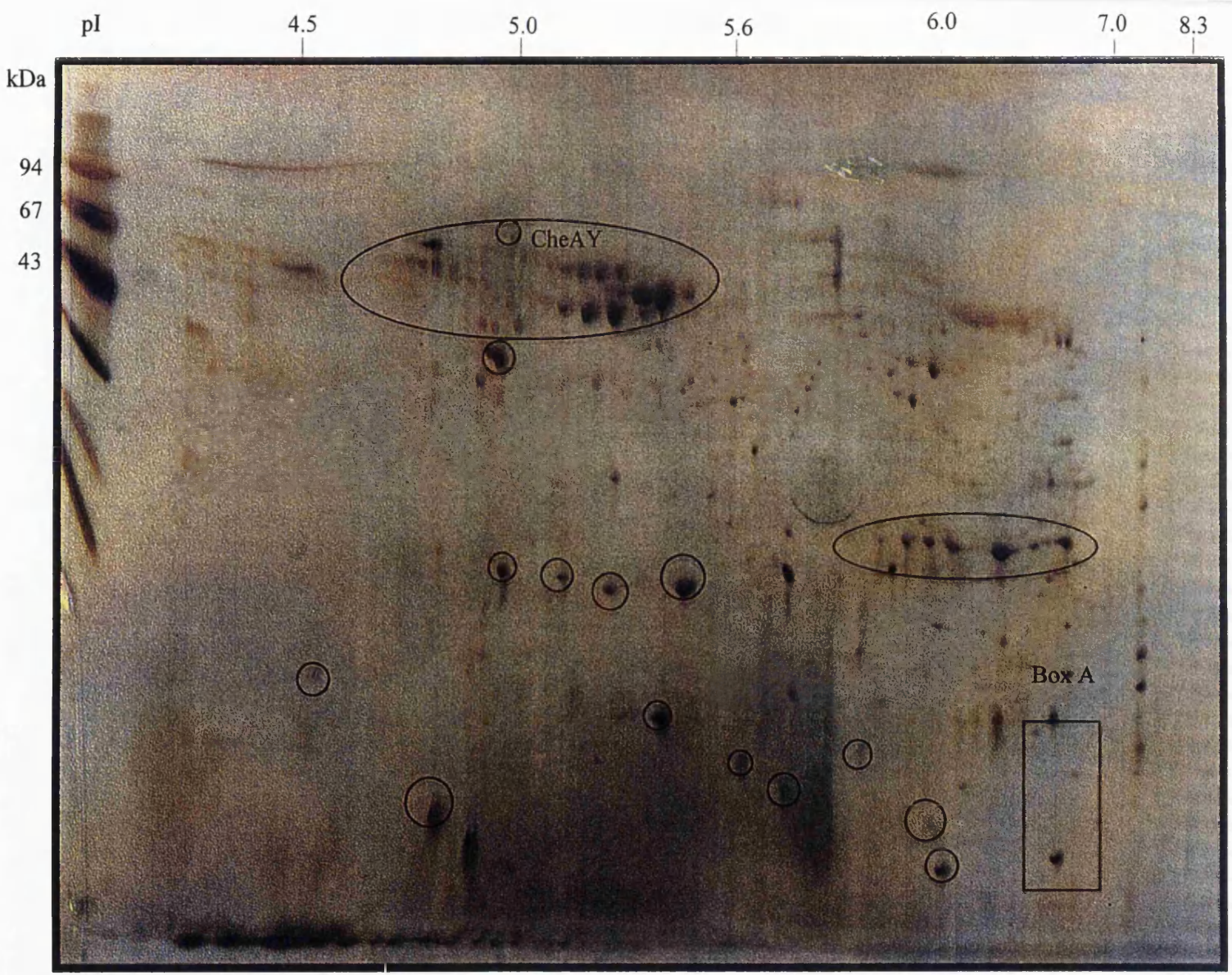


Figure 6.3: 2D SDS PAGE gel of H. pylori N6 cheY1 mutant strain .Circles represent highly conserved proteins. BoxA indicates proteins that may be regulated by CheAY. CheAY is also indicated.

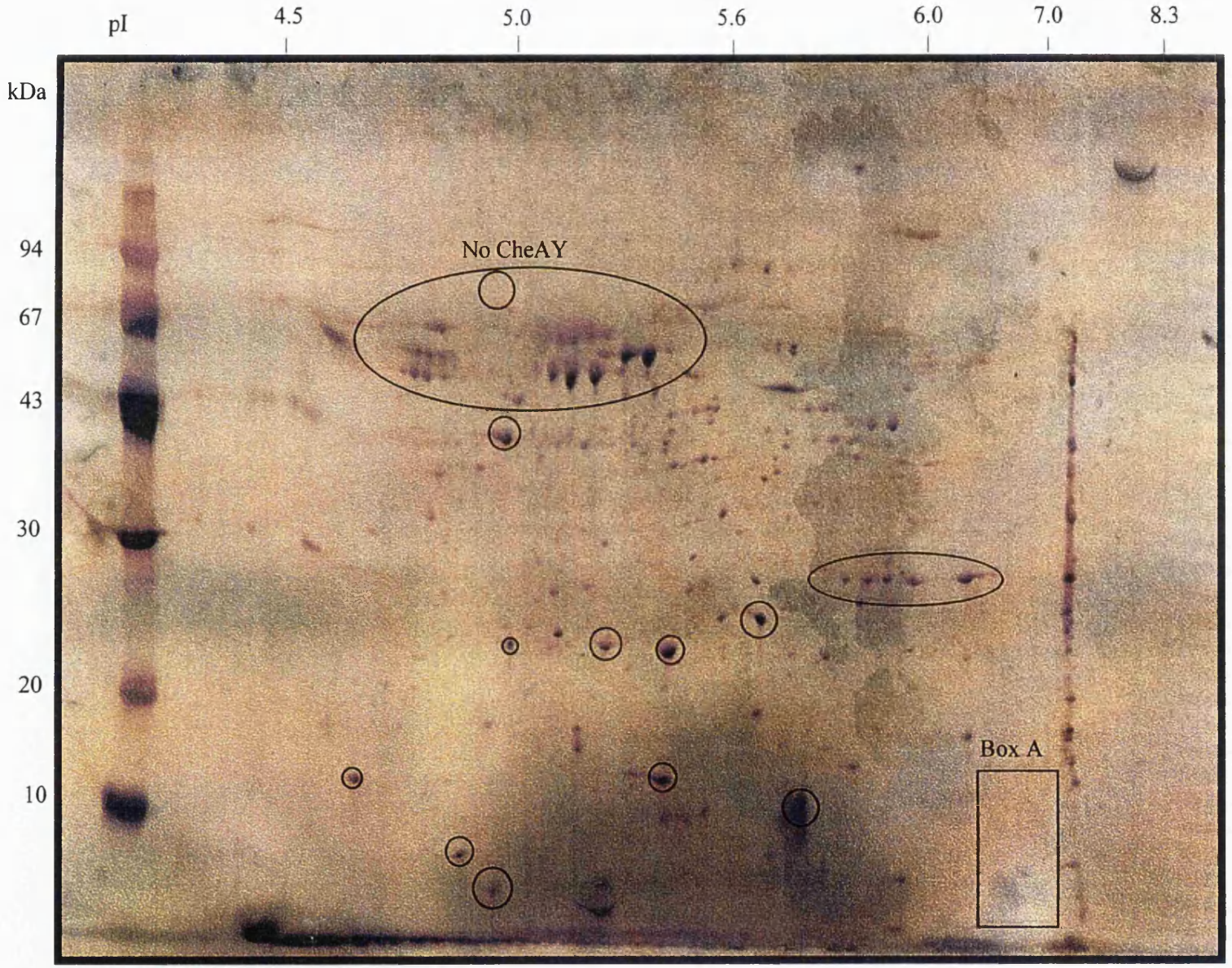


Figure 6.4 : 2D SDS PAGE gel of H. pylori N6 cheAY mutant strain .The putative CheAY spot and the three proteins present in Box A in Figures 6.2 and 6.3 are absent

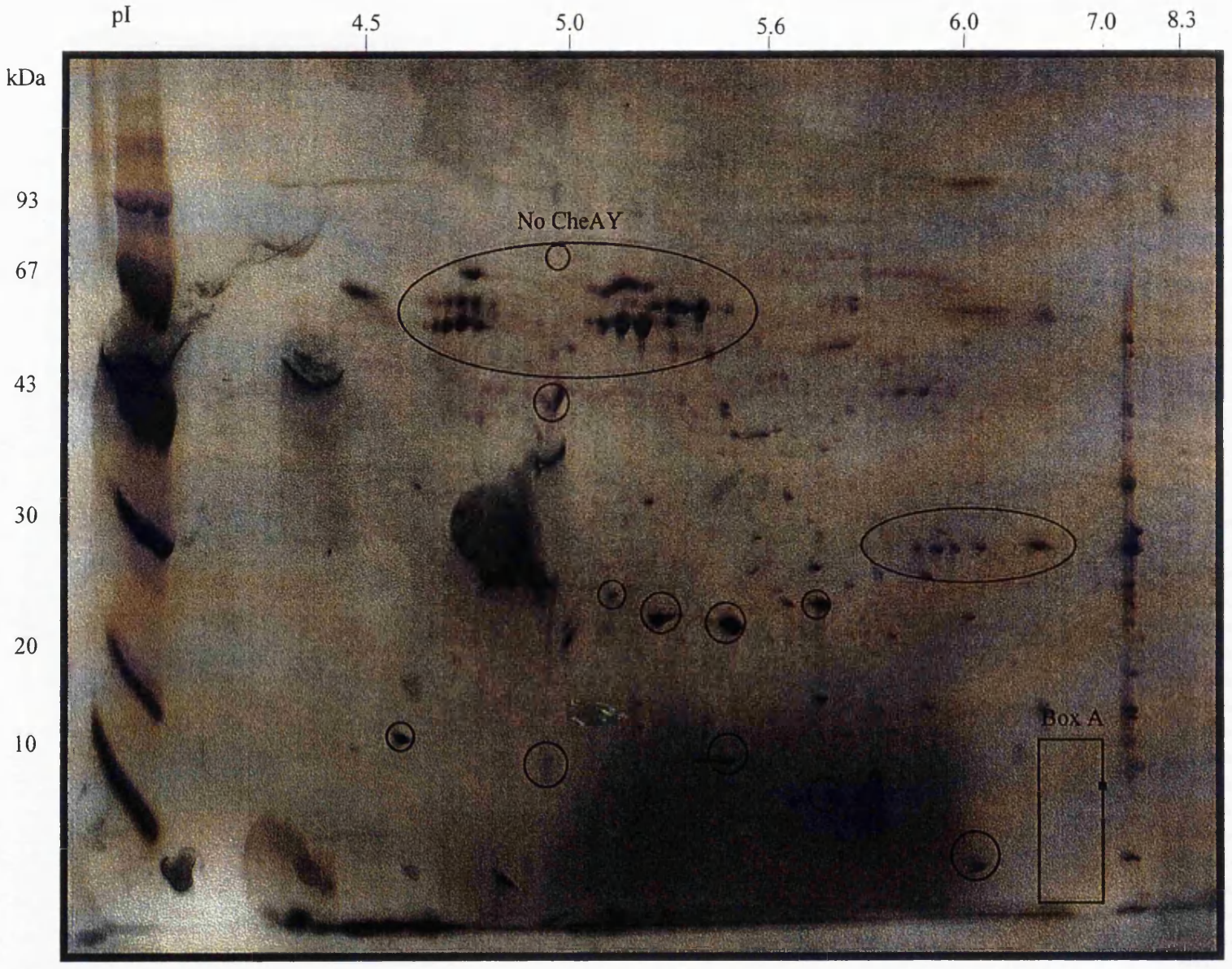


Figure 6.5 : 2D SDS PAGE gel of H. pylori N6 cheAY/Y1 mutant strain .The putative CheAY spot and the three proteins present in Box A in Figures 6.2 and 6.3 are absent

6.2.2.2 Colonisation of mice by *H. pylori* SS1 wildtype and chemotaxis mutant strains.

The results of colonisation of the HSD/ICR mouse model were assesed by a semi-quantitative analysis of *H. pylori* within the stomach (see Section 2.6.5.2). The percent bacterial score for the SS1 strain after 2 and 8 weeks was 58% and 94% respectively. In contrast, neither the SS1ΔCheY1$^-$ or SS1ΔCheAY$^-$ strains colonised any of the 10 mice challenged each (Table 6.2).

Table 6.2 Colonisation of HSD/ICR mice by *H. pylori* SS1 wildtype and cheomtaxis mutant strains.

H. pylori strain	No. infected/total	Challange inocula		Colonisation %	
		Day 1	Day 2	2 weeks	8 weeks
SS1	10/10	$1.05x10^8$	$6.4x10^7$	58%	94%
SS1ΔCheY1$^-$	0/10	$8.9x10^7$	$7x10^7$	0	0
SS1ΔCheAY$^-$	0/10	$1.1x10^8$	$7.9x10^7$	0	0

6.3 Discussion

This chapter describes the use of 1D and 2D SDS PAGE to analyse the protein profiles of the *H. pylori* N6 and SS1 wildtype and their respective chemotaxis mutant strains. The finding that all of the highly expressed proteins, UreA, UreB, FlaA and FlaB were conserved in both the wildtype and mutant strains suggests that the mutagenesis of *cheY*1 and *cheAY* does not affect the expression of abundant *H. pylori* proteins. Additionally, the presence of the FlaA and FlaB proteins in the chemotaxis mutant strains suggests that the phenotypic changes observed were not due to the lack of flagellin, but because of the absence of a fully functional chemotaxis system. Analysis of the 1D PAGE gel profile failed to

identify any band in the 89-90 kDa region which could correspond to the CheAY protein. This is because only highly expressed proteins are visible on a 1D gel. Additionally, proteins less than 20 kDa were not visible on this 12% gel, thus preventing the identification of a CheY1 protein band.

Comparitive analysis of the 2D-PAGE gels of the wildtype *H. pylori* N6 and the chemotaxis mutant strains, ΔCheY1$^-$, ΔCheAY$^-$ and ΔCheAY/Y1$^-$ to putatively identify the position of the CheY1 and CheAY proteins on the proteome of *H. pylori*. The conserved pattern of individual proteins and protein clusters found in the wildtype and mutant 2D gels demonstrates the conserved nature of these profiles for comparative studies. Comparison of gels failed to identify a putative CheY1 protein spot. Based on its location on the 2D protein gel and comparative analysis of the wildtype and chemotaxis mutant gels, a putative protein spot was assigned for the CheAY protein. This could be confirmed by protein sequencing. The absence of three proteins in ΔCheAY$^-$ and ΔCheAY/Y1$^-$ 2D gels which are found conserved in the wildtype and ΔCheY1$^-$ 2D gels, suggests that expression of these three proteins may be under the regulatory control of the histidine kinase domain of CheAY. Protein sequencing by MALDI-TOF analysis or N-terminal sequencing may identify them as some of the five response regulator proteins identified in the annotated *H. pylori* 26695 genome sequence (Tomb *et al* 1997). Such co-ordinate regulation of genes by *H. pylori* may be partly explained by the fact that the *H. pylori* genome contains approximately ten-fold less regulatory genes than *E. coli* (Tomb *et al* 1997). Therefore, such genome organisation may reflect the economy with which *H. pylori* utilises its regulatory genes.

In a study by McAtee *et al* (1998) sequencing of protein spots reactive with purified patient sera, showed that the observed MW and pI values for both the FlaA and FlaB proteins were different to the predicted values. These differences may explain the inability to identify a putative protein spot for CheY1 based on its

predicted MW and pI values. Alternatively the lack of CheY1 Protein spots may be due to the preliminary nature of this work and the quality of the 2D PAGE.

The establishment of 2D reference maps of *Helicobacter* proteins of the total cell or from different subcellular locations e.g outer membrane proteins, should greatly compliment the biochemical and genetic characterisation of *H. pylori* proteins. The combination of the complete sequence of the *H. pylori* genome (Tomb *et al* 1997) and the use of 2D gels to characterise the proteome, should change *H. pylori* research dramatically and allow direct analysis of genes expressed under different conditions and of importance for host-parasite interactions.

In this study the level of colonisation of the gnotobiotic piglets by the parental *H. pylori* N6 strain ($10^3/10^5$ CFU/g of mucosa) was slightly less than that observed in previous studies (Eaton *et al* 1996). No altered pathology in either of the colonised stomachs was observed as visible gastritis is normally only seen from week 2 onwards during the course of infection (Akopyanzs *et al* 1995). Challenge of the HSD/ICR mouse model by *H. pylori* SS1 resulted in colonisation of all ten mice challenged. Mild inflammatory lesions, occasionally accompanied by lymphoid follicle formation, have been observed in mice infected with *H. pylori* SS1 (Ferrero *et al* 1998). However, challenge of the HSD/ICR mouse model with *H. pylori* SS1 resulted in no observed gastritis. This has also been demonstrated for other *H. pylori* strains where, in general, there was little difference from control unchallenged HSD/ICR mice even after several months of colonisation (M^{c}Colm 1997). One possible explanation for the lack of gastritis in the *H. pylori* infected HSD/ICR mouse model is the absence in this host of certain pathways which are involved in the induction of inflammatory responses in humans. For example, the proinflammatory cytokine IL-8, which has been demonstrated to play a role in the host response to *H. pylori* infection (Crabtree *et al* 1994) is absent from mice.

In contrast, the *cheY1*$^-$ mutants of *H. pylori* N6 and SS1 were unable to colonise the gnotobiotic piglet model or the HSD/ICR mouse model, respectively. Mutagenesis of the CheAY homologue in *H. pylori* SS1 also prevented colonisation of the HSD/ICR mouse model. The results obtained in this study correspond to those observed in *C. jejuni*, where mutation of a CheY homologue eliminated the ability to colonise mice or cause disease in ferrets (Yao *et al* 1997). As was discussed in Chapter 5, the chemotaxis mutants of *H. pylori* N6 are motile, but their swimming behaviour is altered, N6ΔCheY1$^-$ exhibits increased tumbling whereas N6ΔCheAY$^-$ swims in straight lines. In addition, their chemotactic response to two known chemoattractants, mucin and urea, was different to that observed for the parental N6 strain.

The inability of the bacteria to alter the directional rotation of the flagella would be expected to affect the ability of the chemotaxis system to cause migration in a controlled fashion towards the mucus lining of the stomach. Viable *H. pylori* were not observed in any of the gnotobiotic piglets challenged with *H. pylori* N6ΔCheY1$^-$ after seven days. Although additional studies with shorter colonisation time points are required to identify how long the bacteria are manitained in the stomach, this suggests that a full chemotactic response is essential for early colonisation, possibly allowing the bacteria to migrate in a controlled manner toward the mucus layer against the gastric flow in the stomach.

Analysis of the parental and chemotaxis mutant *H. pylori* N6 strains by 2D PAGE, identified three putative proteins possibly under the regulatory control of CheAY. The observation that growth characteristics were similar for the mutant and wildtype strains suggests that the lack of regulation these genes by *cheAY* are not significant for bacterial growth and are therefore unlikely affect the colonising ability of the organism.

As *cheY1* and *cheAY* mutations eliminate chemotaxis but not motility, this result directly implicates chemotaxis as a requirement for virulence. Additionally, the

role of these genes in the colonisation process is conserved in both *H. pylori* N6 and SS1 strains.

6.4 Summary

Comparison of the protein profiles of the *H. pylori* N6 and SS1 wildtype and their respective chemotaxis mutants using 1D SDS PAGE gel failed to identify any differences between the strains. A more detailed analysis of the *H. pylori* N6 strain and ΔCheY1$^-$, ΔCheY$^-$, ΔCheAY$^-$, ΔCheAY/Y1$^-$ chemotaxis mutants using 2D SDS PAGE analysis identified three putative proteins possibly under the regulatory control of *cheAY*. Based on the predicted MW and pI values a putative protein spot was identified for CheAY. The chemotaxis mutants of *H. pylori* N6 and SS1 were unable to colonise gnotobiotic piglet and mice animal models. This confirms that the chemotaxis components are essential for colonisation and virulence.

Chapter 7

Conclusion

H. pylori resides mainly in the mucus layer of the stomach or in the intestine in association with areas of gastric metaplasia. The ability to direct, via chemotaxis, the flagellar movement against the gastric flow towards the epithelial cell surface is likely to be important in the colonisation process.

Prior to the initiation of this study, no research had been published regarding the chemotactic motility of *H. pylori*. Flagella regulated motility has been proven to be an essential virulence determinant for the pathogenesis of *H. pylori* (Eaton *et al* 1992). Chemotaxis by *H. pylori* to a trefoil peptide component of mucin, to urea and to Sodium bicarbonate (Turner *et al* 1997, Mizote *et al* 1997, Nakamura *et al* 1998) has recently been demonstrated. Little is known about the molecular mechanism of chemotaxis in *H. pylori*. During the course of this study a *H. pylori* CheY homologue was identified (Beier *et al* 1997), in addition the annotated *H. pylori* 26695 genome sequence, which contains nine putative chemotaxis homologues, was published (Tomb *et al* 1997). As there was little information available on chemotaxis when this project commenced, the initial aims were to identify, clone and sequence *cheY* and *cheA* genes present in *H. pylori* NCTC11637.

One novel response regulator gene was isolated from *H. pylori* using PCRDOP, this was identified by homology, as CheY1 (Chapter 3). Prior to this study no other *Helicobacter* response regulators had been identified. Analysis of the *H. pylori* genome (Tomb *et al* 1997) has identified only three histidine protein kinases, five response regulators and five putative CheY homologues, approximately ten-fold less regulatory genes than is found in *E. coli*. The arrangement of two component systems in *H. pylori* is more similar to *Haemophilis. influenza*, where only four histidine protein kinases and five response regulators have been identified (Fleischmann *et al* 1995). The *H. pylori* genome contains only a small number of response regulators, most of which do not contain the regions of patch homology used in the design of the degenerate

primers. This illustrates the importance of degenerate primer design in PCRDOP experiments, but also demonstrates the power of the PCRDOP technique which isolated the only response regulator protein present containing the regions of patch homology found in the primer combinations. Amplification of *cheY* and conformation of the origin of *cheA* was achieved using sequence information from the annotated *H. pylori* 26695 genome (Chapter 3).

To determine the role these homologues play in chemotaxis, motility and pathogenesis of *H. pylori* N6 and SS1, defined mutants were constructed in *cheY*1, *cheY* and *cheA* using IPCRM and allelic replacement. A *H. pylori* N6 *cheAY/Y*1 double mutant was also constructed (Chapter 4). This study demonstrates that this is a reliable method for constructing defined *H. pylori* mutants. Such an approach will be of particular importance in the post-genomic era as the focus of *H. pylori* pathogenicity research moves away from identification of genes to the determination of gene function.

Computerised tracking showed that *H. pylori* N6 moved with a speed up to 20 μm/s, consistent with that reported by Karim *et al* (1998). The swimming pattern was found to consist of random darting movements, with frequent change in direction and short runs. The N6ΔCheY$^-$, N6ΔCheAY$^-$, and N6ΔCheAY/Y1$^-$ all moved in long straight runs or wide circles with no sharp turns or changes in direction. This suggests that as in *E. coli*, CheY phosphorylated by CheA interacts with the motor resulting in tumbling of *H. pylori* cells. Changes in direction are presumably effected by reversals in the direction of flagellar rotation. This could be further studied by bacterial tethering studies in which the movement of the bacteria, whose flagella is tethered to a slide by antiflagella antibody, is monitored (Sourjik and Schmitt 1996). *H. pylori* shows high amino acid similarity to the chemotaxis components of *E. coli*, therefore it could be proposed that as in *E. coli*, phospho-CheY interacts with the motor to cause clockwise rotation of the flagella and tumbling in *H. pylori*.

N6ΔCheY1$^-$ exhibited a tumbly phenotype closer to that of the wildtype with respect to the frequency of directional changes. Swarming was not observed for the N6ΔCheY1$^-$ strain, therefore the tumbling phenotype is not believed to be due to suppression mutations in *cheY*1. One possibility is that the CheY1 acts as a phosphate sink, accelerating the dephosphorylation of phospho-CheY, therefore helping to terminate the clockwise tumbling response. This hypothesis is consistent with the lack of CheZ homologues in *H. pylori* and the tumbling phenotype of N6ΔCheY1$^-$, similar to the phenotype observed for CheZ mutants in *E. coli* (Hess *et al* 1988).

The reduced response of N6ΔCheY1$^-$ and N6ΔCheAY/Y1$^-$ strains to hog gastric mucin demonstrated the importance of these components in the chemotactic response of *H. pylori* N6 to hog gastric mucin. In contrast the response of N6ΔCheAY$^-$ to both 1% hog gastric mucin and 10 mM urea suggests that this mutated strain can respond to both chemoattractants via an alternative pathway, which may include CheY1 or the CheY domains of the CheV homologues. Alternatively the CheY domains may be directly phosphorylated by small molecules linked to metabolism. This theory is further supported by the finding that the *H. pylori* genome contains in addition to three classical MCP homologues (Tomb *et al* 1997) a soluble MCP homologue (Allan 1997). Therefore, in order to be sensed, attractants could be transported into the cell. This proposed theory co-incides with the study of Nakamura *et al* (1998) which demonstrated that cytoplasmic urease activity was more important than external urease activity in chemotaxis. The repellent response mediated by N6ΔCheAY/Y1$^-$ to optimum concentrations of urea may be mediated by the one of the classical MCP homologues, via an alternative histidine protein kinase or response regulator, in a manner similar to the aerotaxis system of *E. coli*. Why *H. pylori* exhibits a negative response to urea in the absence of its chemotaxis machinery is unknown.

The negative response of N6ΔCheY$^-$ to urea may be due to an imbalance in the phospho-sink, causing a tumbling response.

Two dimensional SDS PAGE analysis identified three putative proteins which may be co-regulated with or under the regulatory control of CheAY. Protein sequencing may identify these proteins as some of the five response regulators, CheV homologues or other virulence related genes involved in adhesion, invasion or flagellar regulated motility which are co-regulated with chemotaxis components.

Mutagenesis of *cheY*1 and *cheAY* prevented *H. pylori* SS1 colonising the mouse, similarly, the defined mutant N6ΔCheY1$^-$ was unable to colonise gnotobiotic piglets. The inability of the mutants to colonise in both animal models demonstrates the importance of chemotaxis in the initial colonisation and pathogenesis of *H. pylori* infection.

Analysis of the *H. pylori* genome found a number of major differences from the *E. coli* paradigm. The presence of three CheV homologues means that there is a total of five potential CheY homologues present in *H. pylori*. These extra CheY domains may function in some of the responses to urea and mucin as described above. Alternatively the CheV domains may also be involved in adaptation, as has been suggested for *B. subtilis*. The lack of CheR and CheB homologues suggests that *H. pylori* may not adapt to changing conditions using the methylation dependent system found in *E. coli*. Adaptation may be regulated by a methylation independent pathway, via the classical MCP homologues with CheV proteins providing a possible adaptation mechanism.

The results of these studies can be combined to produce a model for chemotaxis in *H. pylori*, see Figure 7.1. This model is based primarily on results of experimental work carried out in this study, but also contains a number of unproven hypothesis which require further investigation.

H. pylori swims in random darting movements, with frequent changes in direction and short runs. The chemoattractants, urea and mucin, which are found in epithelial mucus layer of the stomach are sensed externally by the classical MCP's or are transported into the cell were they are sensed by the soluble MCP homologue. The signalling domains form ternary complexes with CheW and CheAY. When the chemoreceptor senses a stimulus it undergoes a conformational change that affects the autophosphorylation activity of CheAY in the ternary complex. In the presence of a positive stimulus, levels of Phospho-CheY are low, and anticlockwise or smooth swimming occurs. In the presence of a repellent the CheA domain of CheAY phosphorylates the CheY domain of CheAY, which in-turn interacts with the flagellar motor switch to cause clockwise rotation or tumbling. The classical MCP homologues, which have membrane spanning regions, probably detect non-imported attractants and also repellents.

CheY1 acts as a phospho-sink for phospho-CheY. CheY1 accepts phosphates from the CheA domain of CheAY and instead of interacting with the flagellar motor, simply hydrolyses the phosphate bond. This serves to terminate the clockwise response quickly and prevent extended periods of counterproductive turning.

A secondary pathway is proposed for chemotaxis to urea and mucin by *H. pylori.* It is proposed to function via alternative histidine protein kinases and response regulators present in the *H. pylori* genome to mediate both positive and negative responses to urea and mucin in the absence of the primary chemotaxis pathway. These responses are probably mediated by the classical MCP homologues whilst the primary pathway is mediated by the soluble MCP which would allow the formation of a soluble complex between the CheW/CheAY and the receptor whilst allowing communication with the polar flagella.

The ability of *H. pylori* to demonstrate chemotaxis towards urea and mucin ensure that the bacteria can migrate in a controlled fashion towards the mucus lining of

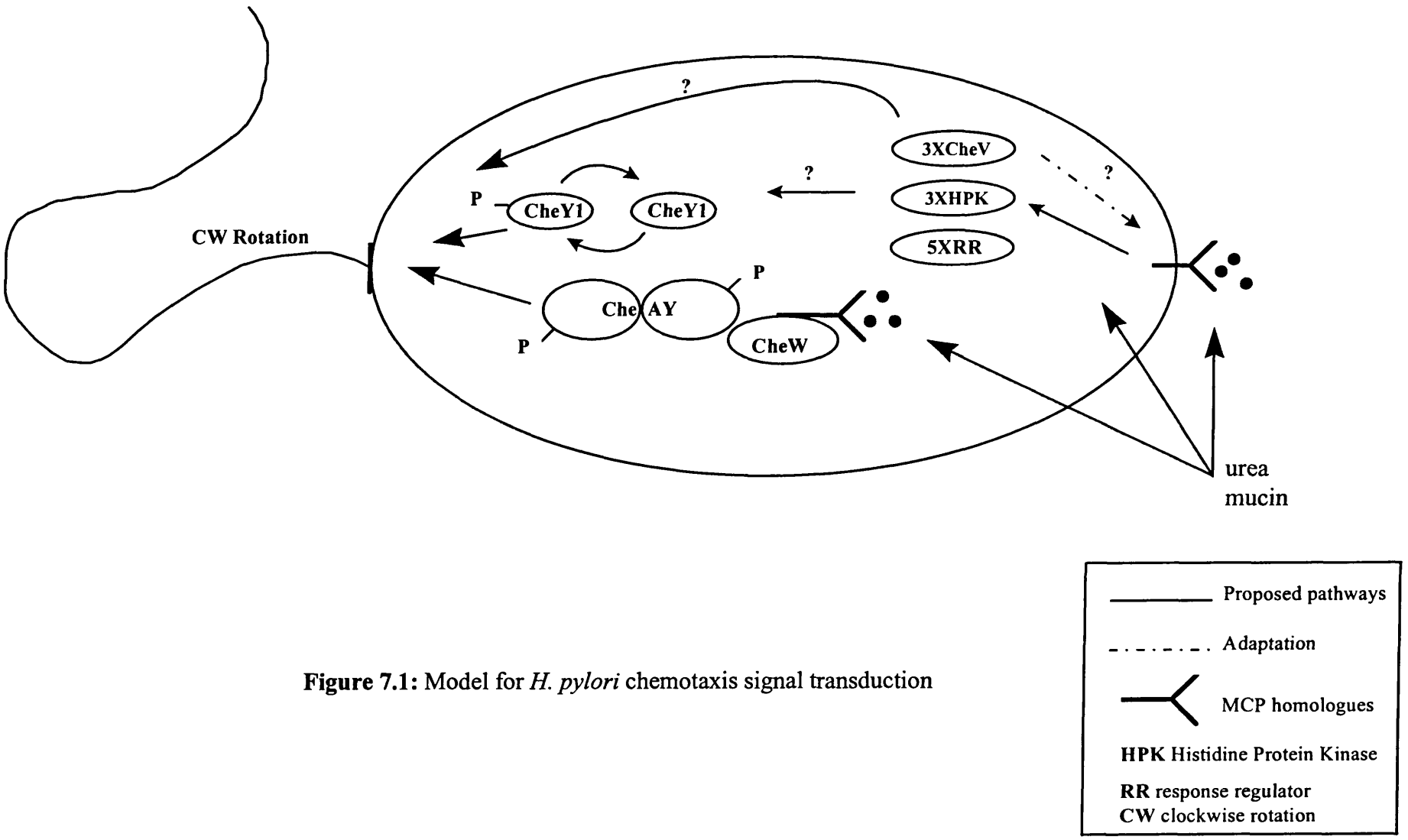


Figure 7.1: Model for *H. pylori* chemotaxis signal transduction

the stomach. Chemotaxis is likely to be essential for early colonisation preventing the bacteria been washed out of the stomach with the gastric flow. Studies on *C. jejuni* have led to the proposal that active motility combined with chemotaxis should be regarded as a potential alternative to specific attachment (Lee *et al* 1986). *H. pylori* adherence to epithelial cells is thought to produce attachment/effacement similar to that seen in the EPEC strains (Segal *et al* 1996). However it has been proposed that only a small proportion of *H. pylori* cells, 1-5%, attach to the epithelial surface (Kirschner and Blaser 1995). Once the host stomach is colonised, motility, known to be essential for *H. pylori* colonisation (Eaton *et al* 1996), combined with a fully functional chemotaxis system may be necessary for the maintenance of non-attached *H. pylori* cells in close proximity to the mucus lining of the stomach.

These results help provide insights into the process of pathogenesis of *H. pylori* infection. It seems clear that chemotaxis in *H. pylori* is essential to enable the bacteria to reach the site of colonisation, penetrate the gastrointestinal mucus and establish prolonged infection by maintaining those bacteria that have not yet colonised in the stomach.

An important line of further work will be to create mutants in the other chemotaxis components, CheW, CheV, membrane bound and soluble MCP homologues, to confirm their roles in *H. pylori* chemotaxis. Of particular interest will be the novel method which *H. pylori* uses to adapt to changing environmental conditions. Additional studies would include identification of the three putative proteins under the regulatory control of CheAY. In *H. pylori*, co-ordinate regulation of chemotaxis with other novel virulence determinants, a system of regulation found in many other bacteria including *V. cholerae*, may exist.

The results of this study demonstrate the importance of chemotaxis and motility in the pathogenicity of *H. pylori* and provide a framework for full elucidation of the chemotaxis system of *H. pylori*.

Appendix Tables 1 and 2: Abbreviations for nucleotide and amino acid sequence

Nucleotide	IUPAC code
A or C	M
A or G	R
A or T	W
C or G	S
C or T	Y
G or T	K
C or G or T	B
A or C or T	H
A or C or G	V
Any	N

Amino acid	Single letter code
Alanine	A
Arginie	R
Asparagine	N
Aspartic acid	D
Asparagine or aspartic acid	B
Cysteine	C
Glutamine	Q
Glutamic acid	E
Glutamine or Glutamic acid	Z
Glycine	G
Histidine	H
Isoleucine	I
Leucine	L
Lysine	K
Methionine	M
Phenylalanine	F
Proline	P
Serine	S
Threonine	T
Tryptophan	W
Tyrosine	Y
Valine	V

Appendix Table 3: List of plasmids used during this study

Plasmid	Description
pUC19	High copy number vector
pBK-CMV	Phagmid rescued from ZAP™ Express lambda vector
pJMK30	*C. coli* 1.4 kb *apha*-3 gene
pILL575	Cosmid Shuttle vector
pCAT	*C. coli* 0.8 kb *cat*GC gene
pCY	pUC19 containing 330 bp RR1
18B	pBK-CMV plasmid containing putative 5 kb *cheY*1 insert
18C	pBK-CMV plasmid containing putative 3 kb *cheY*1 insert
pCY110	pUC19 containing 1 kb insert with total *cheY*1
pCYIP	pCY110 disrupted by IPCRM
pCYIPK1	*apha*-3 gene inserted into the *Bgl*II site of pCYIP, opposite orientation
pCYIPK2	*apha*-3 gene inserted into the *Bgl*II site of pCYIP, forward orientation
pILLCA	pILL57 containing 6 kb DNA fragment with putative *cheA*
pCA110	pUC19 containing 0.7 kb *cheA* gene fragment
pCAIP	pCA110 disrupted by IPCRM
pCAIPK	*apha*-3 gene inserted into the *Bgl*II site of pCAIP, forward orientation
pCAIPc	cat_{GC} gene inserted into the *Bgl*II site of pCAIP
pCF	pUC19 containing 300 bp *cheY*
pCFIP	pCF disrupted by IPCRM
pCFIPK	*apha*-3 gene inserted into the *Bgl*II site of pCFIP

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