A MOLECULAR BIOLOGICAL STUDY ON CAMPYLOBACTER PYLORI

by

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ABSTRACT

C.pylori have been shown to be associated with gastritis and peptic ulceration, but the mechanism of their pathogenicity is unknown. Since a number of virulence factors are known to be plasmid mediated, it was decided to study the plasmids of C.pylori. A variety of techniques were used to establish the best method of plasmid extraction from C.pylori. The method of alkaline lysis as described by Birnboim and Doly was shown to give the most consistent results and the greatest plasmid yield. Plasmid DNA was found in 54% (26 out of 48) of the isolates examined and the plasmids varied in size from 3,4kb to greater than 137kb. The majority (21 out of 26) of isolates had unique plasmid profiles, but 5 isolates showed common ones. Three of these 5 isolates were studied in more detail. The evidence presented here suggests that all 3 plasmid bands visible in these three isolates were different conformations of the same plasmid which has a molecular weight of 6,2 kilobases. The plasmids appeared labile and covalently closed circular DNA was rarely isolated. Restriction enzyme digestion was done with a variety of enzymes, but only 3 of the enzymes used digested the DNA. EcoRI and HindIII partially digested the DNA, while Sau3A digested the plasmids completely, generating 2 fragments of 2,2kb and 2,4kb, and a number of smaller fragments. The DNA was shown to be methylated and the fragments generated by Sau3A digestion suggest that the plasmids may contain a repetitive element. Chromosomal DNA

was also isolated and digested with a variety of enzymes. The chromosomal DNA restriction pattern was shown to be affected by methylation, which may be impor tant when using restriction enzyme patterns to differentiate between strains. Plasmid restriction fragments were end-labelled to detect bands which were poorly visible by ethidium bromide staining. This technique was shown to be more sensitive than ethidium bromide staining of DNA, but the inablility to obtain complete digestion of C.pylori DNA made it impossible to construct a restriction enzyme map of the plasmids. Hybridization experiments showed the plasmids of C.pylori to be related and was also used to detect bands which were not easily visible after ethidium bromide staining. Attempts were made to clone C.pylori DNA into pUC18 and pUC19, but no recombinant plasmids containing C.pylori DNA were obtained.

LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
bp	Base pairs
^{o}C	Degrees Celsius
CIP	Calf Intestinal Phosphatase
cm	Centimetres
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
dpm	disintegrations per minute
dTTP	Deoxythimidine triphosphate
EDTA	Ethylenediaminetetra-acetic acid
EtBr	Ethidium bromide
g	Grams
IPTG	Isopropyl-B-D-thio-galactoside
kb	Kilobase pair
M	Molar
mDa	Megadalton
mg	Milligrams
ml	Millilitres
mM	Millimolar
MOPS	3-[N-Morpholino]propanesulfonic acid
N	Normal
ng	Nanograms

nm	Nanometres
^{32}P ATP	Adenosine triphosphate radioactively labelled with Phosphorous^{-32} in the gamma position
pg	Picograms
RNA	Ribonucleic acid
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
Tris	Tris (hydroxymethyl) amino methane
µCi	Microcurie
µg	Microgram
µl	Microlitre
µM	Micromolar
UV	Ultraviolet
V	Volts
v/v	Volume in volume
w/v	Weight in volume

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CHAPTER 1. INTRODUCTION

1.1 HISTORICAL BACKGROUND

The association of spiral organisms with gastrointestinal disease has been recognised since before the turn of the century, although they have only been identified as C.pylori in the 1980's. According to a review article by Axon (1986), spiral organisms were seen in animal stomachs as far back as the late nineteenth century and were seen in man (at autopsy) in 1906. Work done by Doenges (1939) showed spiral organisms in stomachs of cadavers at post mortem examination, but the organisms could not be cultured. Freedburg and Barron (1940) observed bacteria associated with benign or malignant ulcers in 40% of partial gastrectomy specimens in 1940. Earlier, the presence of urease in the gastric mucosa had been observed (Luck and Seth 1924), but this was not thought to be in any way connected to the presence of the organisms. For many years the presence of urease was attributed to a physiological mechanism for converting urea to ammonia. It was only in 1968 that it was demonstrated that urease was not present in mammalian tissue and that it was a bacterial enzyme (Delluva et al 1968). Despite these earlier observations, the first report of the presence of gram-negative bacteria on the gastric mucosa was made in 1975 (Steer et al). This was largely due to the development of techniques which

allowed gastroenterologists to observe the mucosa of the stomach and duodenum directly. The bacteria were found to be present after ulcer healing and the infiltration of the infected mucosa with polymorphs was noted. Much of the evidence for the association between the presence of the bacteria, the presence of a highly active urease enzyme and the presence of gastritis or peptic ulceration was ignored until the end of the 1970's. This was when Warren noted bacteria present in nearly all biopsy specimens showing histological gastritis. The organisms were labelled "gastric campylobacter-like-organisms" (Warren 1983). The organism was named C.pyloridis later, on the basis of a number of characteristics which are similar to those of other campylobacters (see section 1.3). According to instructions given by the International Code of Bacteriological Nomenclature, the organism was renamed C.pylori recently (Marshall and Goodwin 1987).

1.2 CHARACTERISTIC FEATURES OF C.PYLORI

Warren and Marshall suspected the "gastric campylobacter - like-organisms" were members of the Campylobacter genus on the basis of their spiral morphology when seen in gastric tissue (see Figure 1). This characteristic morphology has been shown to be altered when the organisms are sub-cultured (Buck et al 1986). In pure culture, they are rod-like, and

the spiral shapes are replaced by U-shaped or circular cells. The appearance of these unusual shapes may be as a result of the artificial media used for culture of these organisms. C.pylori are generally 3,5µm long by 1µm wide (Jones et al 1985), have smooth cell surfaces, with 4-6 unipolar/bipolar, sheathed flagella which have terminal bulbs (see Figure 1). These flagella enable the bacteria to maintain their motility in viscous mucous.

Other unique features of C.pylori are their cellular fatty acids, viz: tetradecanoic (14:0);, cis-methylene octadecanoic (19:0); and a small amount of hexadecanoic (Goodwin et al 1985). In addition, the organisms lack methylated menaquinone (Goodwin et al 1985) and most importantly, possess a potent urease enzyme which is present in large amounts. It has been suggested by Goodwin et al (1986) that this enzyme splits the urea which is present in food such as milk and that this results in a cloud of ammonia surrounding the organisms, which protects them from the stomach acid. Biochemically, C.pylori have a number of other unique features. They possess potent extracellular superoxide dismutase and catalase activities and show a strong phosphatase activity with the phenophthalein test (Goodwin et al 1986). They are also hippurate negative, do not ferment carbohydrates and show glutamyl transpeptidase activity (Buck 1988). The biochemical characteristics of C.pylori are summarized in table 1.

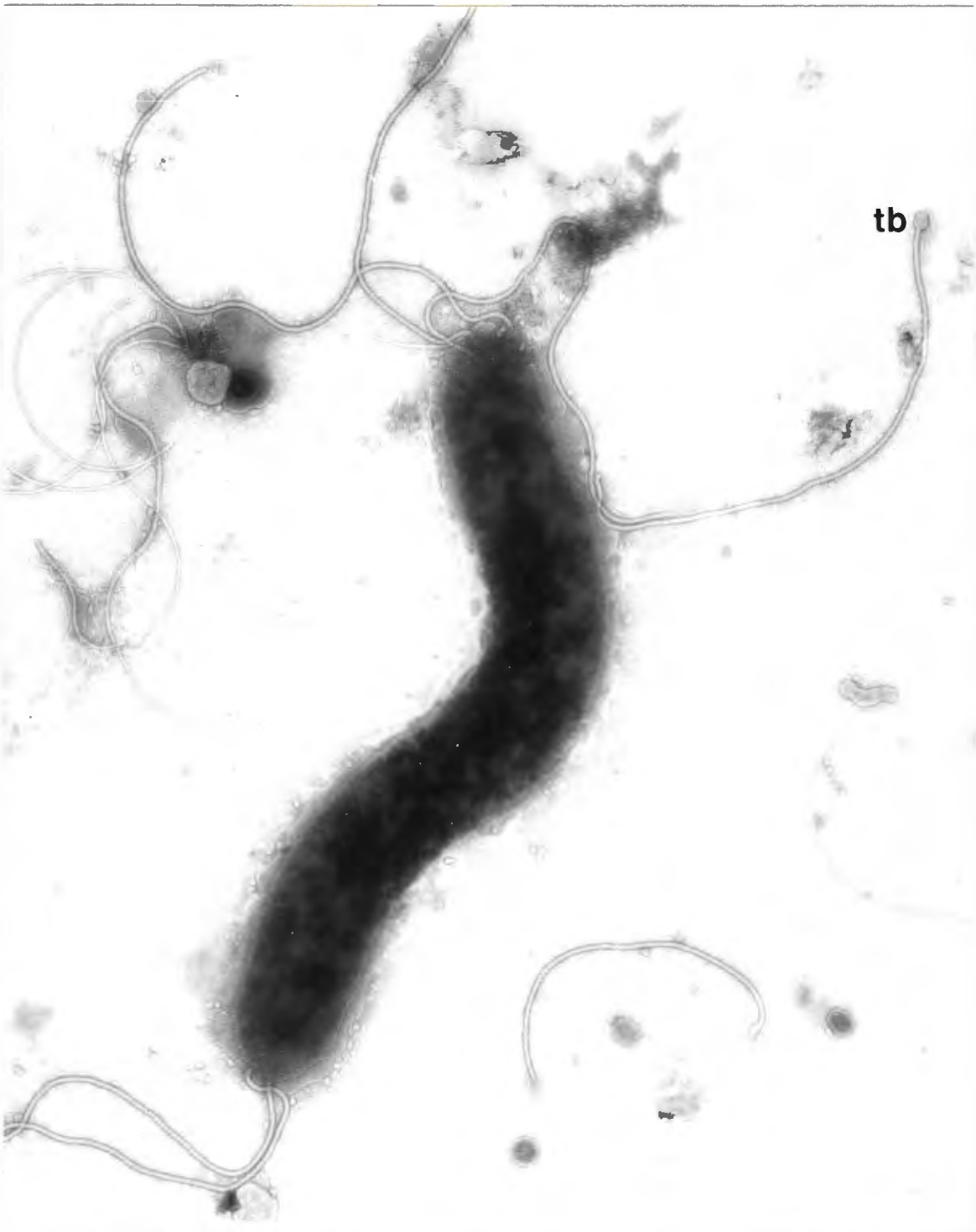


Figure 1: Electron micrograph of C.pylori showing spiral morphology, the polar, sheathed flagella, on one of which a terminal bulb (tb) can be seen. (Original magnification x 7000).

The in vitro susceptibility of C.pylori to antibiotics has been studied. C.pylori is sensitive to a wide range of antibiotics, including: ampicillin; cephalothin; cefoxitin; ciprofloxacin; clindamycin; coumermycin; clorobiocin; kanamycin; nitrofurantoin; novobiocin; penicillin G; erythromycin; tetracycline; tinidazole; metranidazole; and gentamycin, but they are resistant to sulphamethoxazole, trimethoprim, nalidixic acid and vancomycin (Dooley and Cohen 1988, Buck 1988). The anti-ulcer drug, bismuth subcitrate is also effective in eliminating C.pylori, while cimetidine, carbenoloxone, sucralfate and antacid are not.

1.3 COMPARISON OF C.PYLORI WITH OTHER CAMPYLOBACTERS

C.pylori differs biochemically from the other Campylobacters. The high urease, superoxide dismutase and catalase activities distinguish them from the other Campylobacters which have none or low levels of these activities. Another difference between C.pylori and other Campylobacters is revealed by polyacrylamide gel electrophoresis patterns of cytoplasmic proteins, which show differences in protein composition (Pearson et al 1984). The multiple, sheathed flagella with terminal bulbs found in C.pylori differ from those found in the other Campylobacters, which have single, unsheathed flagella that lack a terminal bulb. Most of the Campylobacters have a rough cell wall in contrast to the smooth wall of C.pylori.

The most convincing evidence that C.pylori does not belong to the Campylobacter genus comes from a report on the 16s ribosomal RNA sequences of C.pylori and other Campylobacters (Romaniuk et al 1987). Comparisons of the sequences of these genes from a number of Campylobacter species, indicate that C.pylori is not a member of the Campylobacter genus and is more closely related to Wolinella succinogenes. Marshall (1986) suggests that C.pylori should be viewed as an aberrant Campylobacter that grows in 3 days at 37°C in a humid environment and points out that it has chosen a unique ecological niche where there is no competition from other organisms.

C.pylori share a number of features with members of the Campylobacter genus and these features have been responsible for the classification of the organism in this genus. Firstly, C.pylori grow in microaerophillic conditions (10% CO_2, 5% O_2) at 37°C. According to Buck (1988), some strains will grow at 42°C, but none grow at 25°C. Small, circular, transluscent colonies become visible after 3 days in a humid environment and on a suitable medium (Waghorn 1987). C.pylori do however have slightly different requirements from other members of the Campylobacter genus concerning their growth media requirements (Buck et al 1986), in that they fail to grow on most media without added serum. The majority of published reports claim that C.pylori grow best

on chocolate agar (Buck 1988, Buck and Smith 1987, Morgan et al 1987). Secondly, the morphology of the organism and the genomic DNA base composition of guanine and cytosine (35,8-37,1 mol %) matches that of the other members of the Campylobacter genus.

1.4 DETECTION OF C.PYLORI

1.4.1 EXAMINATION OF BIOPSY SPECIMENS

C.pylori can be detected by a number of procedures. Several biopsy specimens are taken from each patient, and each is handled in the appropriate way for the detection of the organisms. Buck et al (1986) took three biopsy specimens: one was fixed in 10% buffered formalin for light microscopy: another was fixed with 2% gluteraldehyde in 0,2M cacodylate buffer for 2 hours and stored at $4^{o}C$ for electron microscopy; the third was used to inoculate culture media. This specimen was used to inoculate chocolate agar plates and the plates were incubated under microaerophillic conditions. The bacteria were visualised by light microscopy of the sections stained with silver stain, but the Brown-Benn tissue gram stain or the heamatoxylin and eosin stain were used to demonstrate the presence of the bacteria in the tissue as well.

According to Rathbone et al (1986), at least two biopsy specimens are needed to detect C.pylori accurately. They confirm that the Warthin-Starry silver stain is more reliable than the conventional haemotoxylin and eosin stain for detection of the organism. Rathbone et al (1986) used a modified Giemsa stain and found this to be as reliable as the Warthin-Starry stain. They point out that although these methods label all/most of the bacteria present in the biopsy, C.pylori can be identified by its curved morphology and close association with the surface of the enterocytes in the biopsy specimen.

1.4.2 SEROLOGICAL EXAMINATION

C.pylori infection can also be diagnosed by serological assays. In a study done by Perez-Perez et al (1988), they found that IgG and IgA assays were specific for C.pylori, but IgM assays were not. Significantly higher IgG and IgA levels were found in patients with histologically confirmed gastritis and C.pylori infection than those without. The antibody titres appeared to be age related, with IgA and IgG antibodies being found rarely in healthy people under 20 years of age, but 50% of patients older than 60 years showed the presence of antibody.

The specificity of raised titres of IgG and IgA for C.pylori infection was confirmed by Rathbone et al (1986), who

studied antibody titres in serum by an enzyme linked immunosorbent assay (ELISA). In a study of 39 patients with non-ulcer dyspepsia, 20 out of 21 patients (95%) with chronic gastritis and C.pylori infection had significantly raised IgG and IgA antibody titres. Raised IgM antibodies were shown to occur in patients with raised titres to other Campylobacter species, indicating a possible antigenic cross-reactivity of IgM antibodies. In a similar study done by Ireland et al (1986), raised IgG and IgA antibody titres were also shown to be associated with C.pylori infection. Marshall et al (1987) have confirmed that discriminating use of an ELISA antibody test could be of assistance in detecting infection with C.pylori. An interesting study on the detection of C.pylori by serological assay was done by Dwyer et al (1988) in Australia. They determined the C.pylori-specific IgG levels in a group of Aborigines in whom peptic ulceration is virtually unknown. This group of patients showed lower antibody levels than a group of age-matched, healthy, white Australians, who in turn had significantly lower levels of C.pylori-specific IgG than those found in C.pylori positive patients.

The measurement of antibodies against C.pylori have been done using various techniques. These include complement fixation titres, passive haemagglutination tests and ELISAs, the last of which appears to be the most popular technique. Generally, all of the studies have shown raised IgG and IgA

levels in patients infected with C.pylori, but similar titres for IgM in patients with or without C.pylori infection. Raised IgA levels were found, not only in the serum, but also in the gastric juice of colonised patients, indicating a local immune response to C.pylori.

1.5 THE CLINICAL SIGNIFICANCE OF C.PYLORI

C.pylori has been isolated extensively from the gastric mucosa, but its clinical significance and its degree of pathogenicity, if any, remains controversial. There has been a consistent association of the organism with a variety of common upper gastrointestinal tract disorders and this has suggested a possible aetiological role for the organism in some of these disorders.

The association of the organisms with these diseases is, however, not sufficient to prove a causal role for C.pylori in the disease and the question arises as to whether the organism is itself pathogenic or whether it simply is a commensal occupying injured mucosa. There is an increasing amount of evidence which refutes the latter claim. Firstly, the organism is not found in drug-induced antral gastritis (Gustavsson et al 1987). Secondly, the systemic and local antibody responses described earlier suggest that the organism is not a commensal. Thirdly, phagocytosis of the bacteria by polymorphonuclear leukocytes has been

demonstrated in electron micrographs (Shousha et al 1984); this indicates an active response by the host to remove the bacteria. More evidence that C.pylori have a pathogenic role in the gastrointestinal tract comes from the ultrastructural changes associated with the presence of the bacteria in the gastric epithelial cells (Chen 1986). The most convincing evidence for a causal role of C.pylori in histologic gastritis comes from a widely reported self-inoculation experiment by Marshall (1985). Before self-inoculation, the author underwent endoscopy and gastric biopsy, both of which showed normal mucosa and no bacteria. C.pylori (10^9 colony forming units) were swallowed one month later and symptoms were monitored. Transient achlorhydric gastritis was experienced 8 days later, with non-acidic vomiting, epigastric distension, malaise and halitosis. Biopsy specimens taken 10 days after inoculation showed active gastritis with polymorphonuclear cell inflammation in the antrum. Large numbers of C.pylori could be seen and cultured. Other characteristic features of the disease were noted, and included a loss of the pattern of alignment of epithelial cells, irregular bulging surfaces of these cells, a depletion of the microvilli and a severe reduction in the numbers of cytoplasmic mucous secretory granules. Further biopsy specimens were taken on the fourteenth day afer inoculation and these revealed the disappearance of C.pylori and polymorphonuclear cells, and the partial resolution of ultrastructural changes. Treatment was started after the

second endoscopic examination with tinidazole (500mg twice daily) and the symptoms disappeared completely within 24 hours. A second self-inoculation experiment has been reported by Morris et al (1987). They demonstrated acute histologic gastritis of the antrum in association with colonization by C.pylori. In addition, they were able to show persistence of the organism with ongoing gastritis and they treated the patient successfully with a bismuth preparation. These two experiments provide convincing evidence that C.pylori can cause acute histological gastritis which presulmably may become a chronic gastritis.

1.5.1 C.PYLORI AND GASTRITIS

The first recognised association between C.pylori and gastritis was made by Warren in 1979 (Marshall 1986). Nearly all gastric mucosal specimens that he obtained were shown to contain campylobacter-like organisms and most of the specimens had the histological appearance of active chronic gastritis. This included an infiltration with lymphocytes, plasma cells and polymorphs. Marshall and Warren (1984) began a study in 1981 and found that no matter what disease their patients undergoing gastroscopy were suffering from, the bacteria were usually present. They found that 95% of patients with active chronic gastritis had C.pylori infection while infection was rarely seen in histologically normal antral mucosa. Numerous other reports have appeared,

all confirming these results (McNulty and Watson 1984, Rollason et al 1984, Langenberg et al 1984, Phillips et al 1984, Price et al 1985, Pettross 1986, Burnett et al 1984, Ireland et al 1986).

The self inoculation experiments described earlier must be borne in mind when analysing results of an apparent epidemic of achlorhydria in subjects undergoing repeated gastric secretion studies (Ramsay et al 1979). In this study, 17 out of 37 (46%) healthy volunteers became rapidly hypochlorhydric and showed fundal and antral gastritis. The nature of their illness and symptoms were very similar to those described in the self-inoculation experiments and an infectious etiology was suspected in the spread of the illness. This was possible as the pH electrode had not been sterilized between experiments and gastric juice was returned to the stomach after pH measurements were taken. It was stated in a review article by Goodwin et al (1986) that retrospective analysis of the histological specimens showed those with gastritis to have been infected with C.pylori. In a similar gastric secretion study done in Canada (Gledhill et al 1985), 4 out of 6 (67%) previously healthy volunteers developed similar symptoms to those described above. Spiral bacteria were found in specimens from 2 out of 4 (50%) patients, and once again, the pH electrode had not been sterilised between measurements. Epidemic gastritis was thus shown to be associated with infection by C.pylori.

In a study by Buck et al (1986), spiral bacteria identified as C.pylori, were found in 27 out of 39 (69%) of patients with gastritis, but in only 1 out of 7 (14%) patients with histologically normal mucosa. These results demonstrated a distinct association of the bacteria with gastritis, but failed to show a definite association with gastric ulcers. Rollason et al (1984) and Burnett et al (1984) found similar results, but other investigations (Marshall et al 1984, Langenberg et al 1984, McNulty and Watson 1984) have shown that an association between C.pylori and gastric ulcers occurs. Graham and Klein (1987) claim that "evidence for C.pylori as a cause of gastritis is accumulating rapidly." Two types, (A and B), of gastritis occur, and it is type B that has been associated with C.pylori infection. Type B gastritis is more common than type A and affects the pyloric mucosa. Symptoms include inflammation and patchy gastric atrophy. According to Graham and Klein (1987), the causal nature of C.pylori infection with gastritis is supported by: 1) A statistical relationship between type B gastritis and C.pylori infection; 2) a positive correlation between the number of C.pylori organisms and polymorphonuclear cells; 3) a scarcity of polymorphonuclear cells in areas of gastric mucosa not infected with C.pylori; 4) improvement of gastritis after treatment of the C.pylori infection; and 5) the self-inoculation experiments described earlier where the

investigator developed acute gastritis after ingesting a pure culture of C.pylori.

The question remains as to how C.pylori may cause gastritis. According to Waghorn (1987), the bacteria have adapted to be motile in extremely viscous media. They can penetrate and pass freely through the gastric mucous under the influence of chemotactic stimuli and move towards the gastric epithelium. A number of reports (Hazell et al 1986, Waghorn 1987, Wright et al 1988) have stated that the organisms are most often found in the tight junctions between gastric epithelial cells or in the mucous lining the gastric mucosa. The location of the organisms in the intercellular junctions may be as a result of the presence of nutrients or of growth factors such as urea and heamin in this area. The presence of C.pylori attracts neutrophils. This is followed by the development of inflammation and gastritis which is accompanied by the development of adherence pedestals between the organism and epithelial cells. According to Goodwin et al (1986), there is a local thickening of the cell membrane in the area of the adherence pedestal. No particular structures of the bacteria are involved in the adherence. Wright et al (1988) suggest that the bacteria may have a toxic effect on the cells, since dying cells are found along with the bacteria in the tight junctions and in the microvilli. Other effects of the adherence of the bacteria appears to be a splitting and opening up of tight

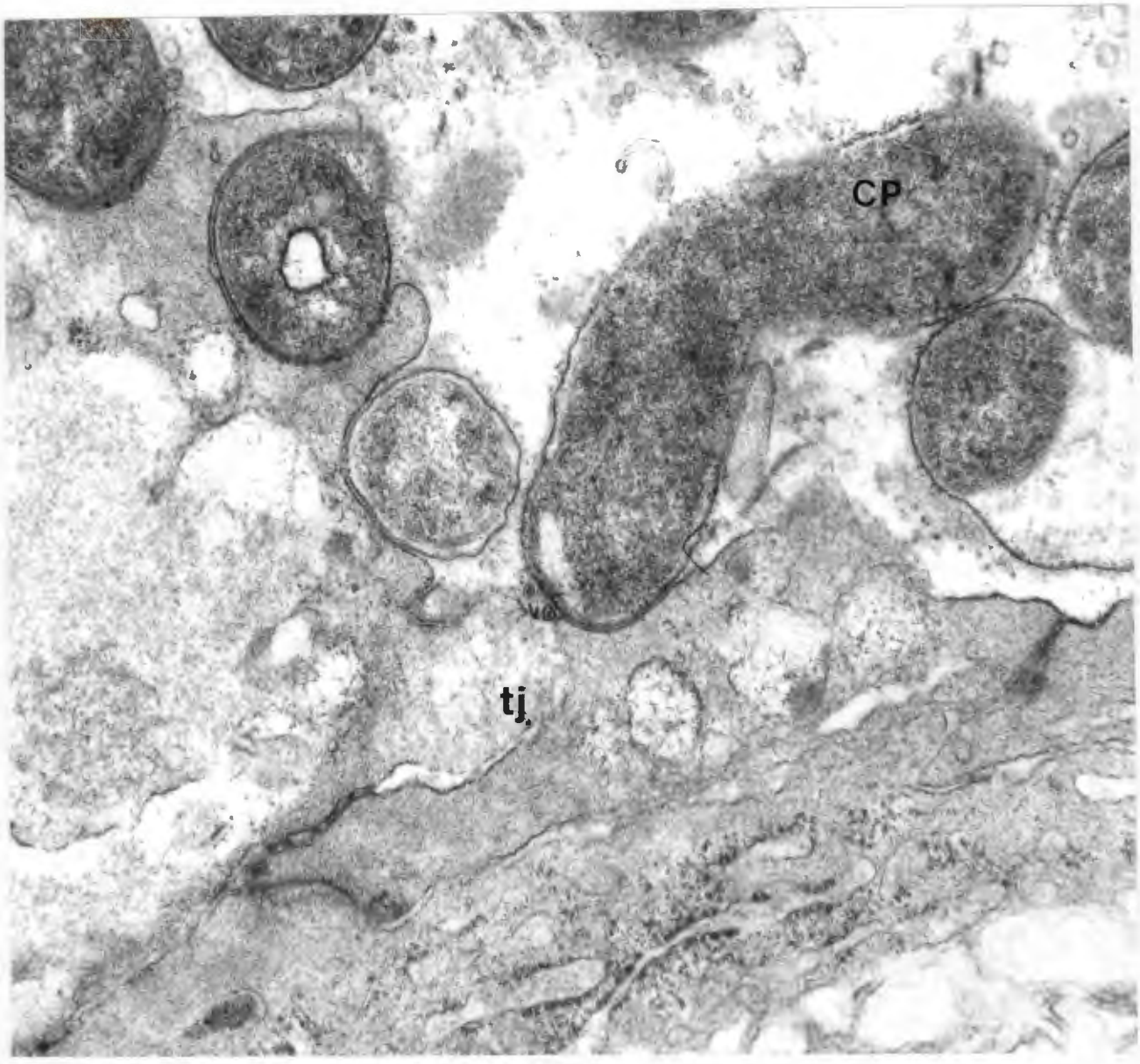


Figure 2: Electron micrograph of an ultrathin section showing C.pylori (CP) at the intercellular tight junction (tj). A number of CP are also embedded into the cytoplasmic membrane. (Lead acetate and uranyl acetate stain; original magnification x 1000).

junctions followed by death of the mucosal cells; depletion of microvilli; disruption of supporting filaments within epithelial cells; decrease in mucous production; and hypochlorhydria. The whole process may take years and this would account for the chronic nature of the disease.

1.5.2 C.PYLORI AND PEPTIC ULCERATION

The association of C.pylori with peptic ulcers is not as well documented as with gastritis, but ulceration would be a logical extension of the infective process. Marshall et al (1985) suggest that damage to the antral epithelium could cause interference with the mucous barrier and allow acid to destroy tissues. Buck (1988) concludes that C.pylori may be one of several predisposing factors that can lead to damage of the gastric epithelium and eventual ulceration. In their study, Hazell and Lee (1986) found that the rapid hydrolysis of urea resulted in the formation of ammonia, which associated with hydrogen ions to form ammonium ions, and produced hypoacidity and toxic effects on the gastric mucosa. Together with a reduction of mucous production, these events lead to ulcer formation when acid and pepsin reach the damaged mucosa. Numerous studies have confirmed the association between C.pylori and duodenal ulcers (McNulty and Watson 1984, Langenberg et al 1984, Pearson et al 1984, Phillips et al 1984). The incidence of C.pylori in patients with duodenal ulcers appears higher than in those

with gastric ulcers. In their review article, Goodwin et al (1986) suggest that chronic inflammation induced by C.pylori may be an important factor in the aetiology of malignant ulcers. It was found by Schrager et al (1967) that patients with ulcers almost always have gastritis. Since strong evidence exists for the pathological role of C.pylori in gastritis, one can speculate about the role of the organism in ulcer formation, which may be seen as an extension of an inflammatory process.

It is likely that a number of factors contribute to the pathogenisis of gastritis and ulcer formation and the evidence presented here suggests that C.pylori may be one of these factors. To understand the possible role of the organism in the pathogenisis of the disease, it is necesary to study its genetic organisation of the organism.

1.6 REASONS FOR STUDYING PLASMIDS IN C.PYLORI

There is much evidence that C.pylori plays a role in the pathogenisis of gastritis and peptic ulcers, but of what significance is the study of the plasmids in this organism?

The information gained can elucidate the contribution of the plasmid to the cell phenotype and allows the comparison of the properties of the plasmids from the same or related species. The characterization of the plasmids leads to an

understanding of their epidemiology, evolution and particularly, to their contribution to the unique attributes displayed by bacteria. Plasmid profiles themselves, when compared with those from different isolates of the same species, can be of great epidemiological value (Schaberg et al 1981). Tenover et al (1985) have already shown that plasmid fingerprinting is of value in tracing the epidemiological spread of Campylobacter jejuni infections.

The investigation of C.pylori by genetic methods may reveal that plasmids carry resistance factors which determine the antibiotic resistance phenotype of their host organisms and which may be clinically important. If the antibiotic resistance genes are plasmid borne, then this will have epidemiological importance in a clinical setting. Plasmids are often self-transmissable and may be transferred to other organisms, thus rendering these organisms resistant to the same antibiotics. Other important factors which may be plasmid borne are metabolic properties which may determine the ability of the host bacterium to survive under specific conditions.

The most important reason for studying the plasmids of C.pylori is that a number of virulence factors are known to be plasmid mediated in a variety of bacteria. These factors include: adherence to epithelial cells; enterotoxin production; invasiveness; the presence of colonization

factor antigen; resistance to complement and antimicrobial compounds; and the ability to accumulate iron.(Walker et al 1986). These will be discussed later in more detail.

Plasmids also carry incompatibility and exclusion properties. These properties determine whether certain plasmids are able to co-exist in the same cell line. Generally, incompatibility between two plasmids is a measure of their similarity or genetic relatedness, such that the more closely related two plasmids are, the greater their incompatibility and the less stable they will be in the same cell line. In gram negative bacteria, conjugative plasmids encode sex pili of a particular incompatibility group which confer specific adsorption specificities for phages to the pili (Stanisich 1984).

Plasmids therefore confer many properties on their host cells. Since nothing is known about the mechanism of pathogenicity of C.pylori, the study of the plasmids in this organism may be of value to our understanding of the pathogenicity of these organisms.

1.6.1 THE STUDY OF PLASMIDS IN EPIDEMIOLOGY

The value of plasmid studies in tracing the epidemiological spread of infective disease has already been mentioned. Identical plasmid profiles may indicate the spread of the infective organism via person to person contact or from a common source. In C.pylori, this would be valuable information as the mechanism of transmission or source of infection is unknown.

Plasmid profiles can also be of value in the classification of organisms which are difficult to serotype. Several serotyping schemes have been described for C.jejuni or C.coli, but certain strains are difficult to differentiate by this technique. Differentiation of strains on the basis of biochemical reactions is also difficult because only a few biochemical reactions can be detected (Bradbury et al 1983). In a study done on C.jejuni and C.coli, Bradbury et al (1984) found that plasmid profiles were different for individual strains which had been differentiated by serotyping. This finding confirmed the usefulness of plasmid profiles for fingerprinting strains in epidemiological studies. Bopp et al (1985) also did a study to evaluate the usefulness of antimicrobial susceptibility and plasmid profiles as markers in epidemiological studies and found that although seroptyping gave the best results when investigating outbreaks of C.jejuni infections, a

combination of this with either plasmid profile analysis or antibiotic susceptibility patterns gave the most information about the spread of the organisms.

At present, no biotyping or serotyping system exists for C.pylori. This prevents any epidemiological studies from being done. These studies are of particular interest because many patients appear to relapse soon after apparently successful antibacterial treatment. Medical staff need to know whether the relapse is due to recrudescence following incomplete eradication of bacteria or to reinfection by new organisms. In a study by Langenberg et al (1986), isolates were differentiated by their chromosomal restriction enzyme patterns after digestion with HindIII. Although isolates appeared to belong to a homogeneous group on the basis of their microscopic morphology and biochemical and growth characteristics, isolates could be differentiated on the basis of their chromosomal restriction enzyme digest patterns. A similar technique could be applied to plasmid DNA where the plasmid restriction enzyme patterns of isolates could be used to differentiate between strains. The presence of identical plasmids in different isolates would indicate that the transfer of plasmids between isolates had occurred.

Kung et al (1988) have differentiated three biotypes of C.pylori with an API ZYM test kit, but it remains to be seen whether this technique will be a useful diagnostic tool.

1.6.2 PLASMID MEDIATED VIRULENCE FACTORS

A number of plasmid mediated virulence factors have already been mentioned. Some of these, namely adherence proteins and toxin production, have been extensively studied in certain organisms, while their pathogenic role in other organisms is merely speculative and based on the association between the presence of plasmids and virulence. The well-described plasmid mediated virulence factors will be discussed in more detail.

1.6.2.i) VIRULENCE PLASMIDS OF ENTEROPATHOGENIC ESCHERICHIA COLI

Enteropathogenic E.coli have been identified as causative agents of diarrheal disease (Elwell and Shipley 1980). The organisms isolated from ill people were shown to be capable of elaborating one or both of two types of toxin, while those from healthy people could not. One of the toxins, LT (heat labile), is similar to the cholera toxin, while the other, ST (heat stable), is not. The enteropathogenicity of these E.coli strains is not only due to toxin production, but also due to their ability to colonize intestinal

epithelial cells. Surface antigens, called colonization/adherence antigens are responsible for this colonization and not only do they enhance retention and proliferation of these E.coli in the intestine, but they also promote heamagglutination of animal /human erythrocytes. Varying patterns of adherence (localized and diffuse adherence) have been been shown to be encoded by different plasmids (Nataro et al 1985).

The incidence of conjugative plasmids is higher in enterotoxigenic E.coli strains than in E.coli strains isolated from healthy people (Gyles et al 1974, Smith and Linggood 1970). The genetic determinants for enterotoxin production and surface antigens have been shown to be plasmid mediated by experiments indicating that these two factors are self-transmissible. In addition to plasmids carrying enterotoxin and colonization antigen determinants, enterotoxigenic E.coli strains often carry a plasmid mediating antibiotic resistance.

The knowledge that these two major factors (toxin production and adherence antigens) are plasmid borne, is of value when considering mechanisms of pathogenicity of other organisms.

1.6.2.ii) VIRULENCE PLASMIDS OF INVASIVE ESCHERICHIA COLI

Invasive E.coli strains cause generalized, intestinal infections in man and animals. These infections cause invasive diarrheal disease which clinically resemble shigellosis (Small and Falkow 1988). Minshew et al (1978) showed that E.coli implicated in extra-intestinal infections were more likely to a) be hemolytic, b) produce colicin V (ColV), c) hemagglutinate red blood cells in the presence of D-mannose, d) kill 13 day old chicken embryos, than were randomly picked fecal isolates. Hemolysin production and colicin biosynthesis are known to be plasmid mediated in E.coli (Helinski 1973, Novick 1969). ColV plasmids have been extensively studied. The production of ColV has been associated with E.coli strains which cause bacteraemia. E.coli strains harbouring ColV plasmids also have a greater ability to survive in the blood, peritoneal fluid and alimentary tracts of experimental animals. The determinants for serum resistance, increased virulence and ColV biosynthesis have been located by cloning restriction enzyme fragments of the plasmid. ColV plasmids have also been shown to specify a novel iron uptake system that contributes to the pathogenicity of invasive E.coli strains. This increased capacity to scavenge iron and to resist the lethal effects of serum are important aspects of the invasive property of some ColV plasmid-containing E.coli strains. The general concensus indicates that several plasmid-mediated

determinants are necessary for the expression of the invasive phenotype. Small and Falkow (1988) have recently identified a 25kb BamHI fragment on a 230kb plasmid from enteroinvasive E. coli which is required for epithelial cell invasion. Once again, the genetics of the invasive phenotype is complex and the authors suggest that two or three regions within this fragment are necessary for pathogenisis.

1.6.2.iii) R PLASMIDS AS VIRULENCE FACTORS

R plasmids carry antibiotic resistance determinants and are of great importance in clinical settings. However, it has been speculated that these plasmids may carry virulence enhancement factors in addition to antibiotic resistance determinants. In their review article, Elwell and Shipley (1980) state that it is difficult to quantify the concept of virulence and some confusion appears to exist as to whether antibiotic resistance itself can be considered a virulence factor. Little convincing evidence exists for the theory that R plasmids per se increase the virulence of their host bacteria. The emergence of multiply drug resistant enteric pathogens definitely compromises medical treatment and can result in dramatically high morbidity and mortality.

1.6.3 VIRULENCE FACTORS IN C.JEJUNI AND C.COLI

Although C.jejuni exhibits many virulence factors known to be plasmid mediated in a variety of bacteria (adherence, invasiveness, toxin production, iron sequestration, serum resistance and resistance to antimicrobial compounds), only antibiotic resistance and enterotoxin production have been shown to be plasmid mediated in these bacteria.

1.6.3.i) ANTIBIOTIC RESISTANCE

A variety of tetracycline resistant plasmids have been described in C.jejuni. These reports (Tenover et al 1980, Taylor et al 1981, Tenover et al 1985) describe tetracycline resistance plasmids ranging in size from 2-162kb, yet despite the differences in molecular mass and restriction enzyme patterns, the plasmids show a high degree of sequence similarity. An interesting point about the tetracycline resistance determinant from these plasmids is that the one that has been cloned lacks homology to the E.coli tetracycline resistance gene, suggesting a different evolutionary origin of resistance genes in C.jejuni (Walker et al 1986).

Kanamycin resistance genes have also been studied in C.coli. A kanamycin resistance gene encoded on a plasmid also harbouring a tetracycline resistance gene, has been shown to

be a 3'-aminoglycoside phosphotransferase of type III, which has never been identified in gram negative bacteria before (Lambert *et al* 1985). The nucleotide sequence of this gene is almost identical to those of the kanamycin resistance genes in *Streptococcus* and *Staphylococcus* species and suggests that the gene may have been transferred from the gram positive species to the gram negative *Campylobacter*.

1.6.3.ii) TOXIN PRODUCTION

C.jejuni is known to produce at least two exotoxins: a heat labile enterotoxin (CJT) and a cytotoxin. It is not known which specific symptom of diarrheal disease is caused by each of these toxins. The *Campylobacter* enterotoxin has been reported by several workers (Klipstein and Engert 1984, McCardell *et al* 1984, Ruiz-Palacois 1983) and has recently been purified to a greater extent by McCardell *et al* (1987) and Torres *et al* (1987). McCardell *et al* (1987) showed it to consist of two subunits with a molecular weight of approximately 60 000 and 15 000 daltons. The activity of both subunits was neutralized by rabbit antiserum to cholera toxin, indicating an immunological relatedness between the two toxins. The enterotoxin is thought to be responsible for watery diarrhea in *Campylobacter* infected patients and has been shown to be similar to the cholera toxin in many respects. The enzymatic activity of CJT is similar to that of the cholera toxin (CT) and the heat labile toxin of

E.coli (LT). McCardell and Madden (1985) have shown that CJT production is optimal in the presence of iron at a concentration greater than that required for growth of the organism, indicating that a possible specialised iron uptake system is required for virulence expression.

Calva et al (1987) studied DNA sequence homology between CJT and a region of the B subunit of LT and CT. They found a partial DNA sequence homology between the genomic DNA of C.jejuni, LT and CT. This homology was found in toxigenic and non-toxigenic strains and the authors suggest that the enterotoxin genes are a part of the structural genomic DNA in all Campylobacters, and unknown regulatory machanisms participate in their expression; or non-enterotoxigenic strains produce an incomplete subunit containing only the B subunit fragment.

Are these enterotoxins plasmid encoded? Lee et al (1985) found that 18 out of 30 (61%) enterotoxigenic C.jejuni isolates contained plasmid DNA, indicating that the presence of plasmids does not always correlate with enterotoxin production. Indeed, as was described earlier, the sequence homology with LT was chromosomally located, but it is possible that the control of expression of these genes is plasmid mediated. Lee et al (1985) were able to demonstrate that toxin production could be conjugally transferred by plasmid pGK103, together with tetracycline resistance in 2

clinical isolates from distinct areas. This suggested that these plasmids either encode the structural genes for toxin production, or contain regulatory sequences capable of turning on toxin production.

Recently, Taylor et al (1987) studied two 45kb tetracylcine resistance plasmids to identify plasmid encoded/mediated cytotoxin or enterotoxin activity. Plasmid pUA466 was isolated from the same host bacterium as that described by Lee et al (1985) and corresponded to plasmid pGK103. Conjugation experiments were done to transfer plasmids between various Campylobacter strains. Cytotoxin and enterotoxin activity of the organisms were determined using Vero cells and Chinese hamster ovary cells respectively. Taylor et al were able to show that pUA466 and pMAK175, a plasmid from a C.jejuni strain producing both cytotoxin and enterotoxin, did not show cytotoxin or enterotoxin activity. The authors give two possible explanations for their results. The first is that the transconjugants were not true transconjugants, but spontaneous nalidixic acid (used in the selection of transconjugants) resistant mutants. The other explanation for these results is that C.jejuni plasmids may acquire regions of Campylobacter chromosomal DNA by recombination. In this process, toxin determinants could be acquired from the chromosome and co-transferred with a plasmid. It is not known whether and how the plasmids could mobilize toxin determinants from the chromosome of one

C.jejuni strain to a second strain. The theory of recombination between plasmids and chromosome is supported by experimental evidence from Tenover et al (1985) which shows that a tetracycline resistance plasmid showed homology with chromosomal DNA from a tetracycline susceptible, plasmid-free C.jejuni strain. Plasmid pGK103 isolated by Lee et al (1985) also showed homology with chromosomal DNA from an enterotoxigenic, tetracycline susceptible, plasmid-free strain. Taylor et al (1987) also report DNA homology between a tetracylcine susceptible mutant of pUA466 and chromosomal DNA from a tetracycline susceptible C.jejuni strain. Walker et al (1986) confirmed the possibilty that Campylobacter plasmids may be capable of recombining with regions of the chromosome and of mobilizing the chromosomal sequences into other strains.

Johnson and Lior (1987) have described a cytotoxic factor present in many culture filtrates of Campylobacter strains. The toxin was termed a "cytolethal distending toxin" as a result of its capacity to cause progressive cell distension and eventual cell death. It had a molecular weight of over 30 000 daltons and was found in 41% of 718 strains that were tested. The toxin could not be neutralized by anti-cholera toxin antibodies and no association of toxigenicity with serotype or biotype of the organisms could be established.

1.6.3.iii) INVASIVENESS OF C.JEJUNI AND C.COLI

The invasive potential of C.jejuni has been established and may be a possible factor in the pathogenicity of the organisms. Histological, immunohistochemical and electron microscopic examination of biopsy specimens have demonstrated that the pathogenisis of Campylobacter enteritis involves invasion of the intestinal mucosa (Van Spreeuwel 1985). Bukholm and Kupperud (1987) examined the ability of C.jejuni and C.coli to invade epithelial cells in vitro. They found that enteroinvasive Salmonella, Shigella and E.coli exerted an effect which rendered C.jejuni capable of intracellular localization in epithelial cells. None of the Campylobacter cells were capable of infecting the epithelial cells in the absence of either the Salmonella, Shigella or E.coli bacteria. The authors note that the invasiveness of Salmonella, Shigella and E.coli is the result of endocytosis of the bacteria by the host cell. This endocytosis is a result of specific information carried by the bacteria. They point out that in Shigella and E.coli this information is located on plasmids, but make no mention of whether the Campylobacter strains contained plasmids. The mechanism of invasion was shown to be different from that of S.typhimurium in that invasion required active Campylobacter metabolism. Not all co-infecting cells containing plasmids were able to induce internalization of C.jejuni and one co-infecting E.coli strain without plasmids could induce

internalization of C.jejuni. This raises the question as to whether plasmids played any part in this apparent synergistic interaction.

1.6.4 PLASMIDS IN C.PYLORI

There are a limited number of reports on the isolation of plasmids from C.pylori. Majewski and Goodwin (1988) did a study on restriction enzyme analysis of the genome of C.pylori and noted plasmids on about 50% of their isolates. Plasmids were found in a similar number (58,5%) of West Australian isolates (Tjia et al 1987). It appeared that most isolates had unique plasmid profiles and the plasmids varied extensively in size (1,8kb-40kb). No phenotype could be ascribed to any of the plasmids, but Tjia et al (1987) confirmed that plasmid profiles could be useful in distinguishing different isolates and in epidemiological studies. Plasmid profile analysis, when used in conjunction with genomic restriction enzyme analysis, indicated that different strains could exist in the same patient.

The author of this thesis found plasmids in 48% of isolates examined (Penfold et al 1988). This figure has recently been updated to 54%. Three of the original 42 isolates examined had identical plasmid profiles, while the remainder had unique profiles. The plasmids examined in this study also

varied in size, from 2,4mDa to greater than 96mDa (3,4kb-137kb).

The limited studies done on the plasmids of C.pylori and the potential for identifying possible virulence factors associated with these plasmids were two of the reasons for this study.

CHAPTER 2 MATERIALS

2.1 MEDIA AND SOLUTIONS

Boiled blood agar, 2% blood agar, Luria agar and Luria broth were supplied by the Department of Medical Microbiology, University of Cape Town. They were prepared as follows:

1) 2% Blood Agar:

Proteose Peptone	15,0g
Liver Digest	2,5g
Yeast Extract	5,0g
Sodium Chloride	5,0g
Agar	12,0g

The solution was made to a total volume of 1 litre and heated to 50^{o}C and blood was added to a final concentration of 2%.

2) Boiled Blood Agar: This was made up in the same way as 2% blood agar, but the solution was heated to 70^{o}C before the blood was added, so that the cells lysed.

3) Luria Agar:

Tryptone	10,0g
Yeast Extract	5,0g
Sodium Chloride	10,0g
Difco Agar	12,0g

The solution was made to pH7,2 and the total volume was 1 litre.

4) Luria Broth:

Tryptone	10,0g
Yeast Extract	5,0g
Sodium Chloride	5,0g

The solution was made to pH7,4 with NaOH in a total volume of 1 litre.

Tryptose blood agar was supplied by the Department of Microbiology, Red Cross Children's Hospital, Cape Town and was made up as follows:

5) Tryptose Blood Agar:

Tryptose	10,0g
'Lab Lemco Powder'	3,0g
Sodium Chloride	5,0g
Agar	12,0g

Unlysed horse blood (5%) was added and the solution was made to pH7,2 in a total volume of 1 litre.

All the broth and agar solutions were sterilized by heating to 115^{o}C for 15 minutes and were stored at room temperature.

Antibiotics: Ampicillin was stored in aliquots (25mg/ml) at -20^{o}C.

Phenol: Phenol was melted at 56°C and aliquots were stored at -20°C. For use, it was remelted and 8-hydroxyquinolone (final concentration 0,1%) was added. The molten phenol was extracted several times with 1M Tris-HCl (pH8,0) until the pH of the solution was below pH7,6. B-mercaptoethanol (0.2%) was added and the solution was stored under 1M Tris-HCl (pH8,0) at 4°C.

Chloroform/isoamylalcohol: This was used as a solution of 24:1 (chloroform/isoamylalcohol) and stored at room temperature.

2.2 ENZYMES AND KITS

Enzymes were supplied by Amersham International plc, Amersham Place, Little Chalfort, Buckinghamshire, England, HP7 9NA; Bethesda Research Laboratories (BRL), Gaithersburg, Maryland 20877; and Boehringer Mannheim GmbH, Biochemica, P.O. Box 310 120, D-6800, Mannheim 31, West Germany.

Amersham supplied the following enzymes: BglI, HaeII, HpaI and Klenow DNA Polymerase. Boehringer Mannheim supplied Calf Intestinal Phosphatase, NcoI, SmaI, T_4 DNA Ligase and XbaI. BRL supplied AvaI, BamHI, BglII, EcoRI, HindIII, MboI, PstI and Sau3A. Nick translation Kits and Multiprime Kits were supplied by Amersham.

2.3 CHEMICALS, REAGENTS AND OTHER MATERIALS

BDH, Poole, England were the suppliers of B-mercaptoethanol, dimethylformamide, EDTA, ethanol, isopropanol, HCl, NaOH, phenol, potassium acetate, sarkosyl, sucrose and triton.

Boehringer provided caesium chloride, IPTG, lysozyme and Tris.

Merck, Frankfurter Strasse 250, D-6100. Darmstadt, Germany supplied acetic acid, chloroform, ethidium bromide, formamide, 8-hydroxyquinolone, isoamylalcohol and NaCl.

The following were obtained from Sigma, P.O. Box 14508, St. Louis, MO 63178, USA: Bromophenol blue, Brij58, lithium chloride, MOPS, SDS, X-GAL and xylene cyanol.

Formic Acid was supplied by Univar, Saarchem pty, ltd., P.O. Box 144, Muldersdrif, 1747, South Africa.

Amersham supplied Hybond-N and ^{32}P[dCTP], while ampicillin came from Beechams. Sephadex G-50 was obtained from Pharmacia, Fine Chemicals AB, Uppsala, Sweden and Seakem GTG Agarose as well as low gelling temperature agarose was supplied by FMC Corporation, Marine Colloids Division, Rocklands, ME 04841, USA.

Spectrum Medical Industries, Inc. 60916, Terminal Annex, Los Angeles 90054 were the suppliers of Dialysis tubing and Oxoid Limited, Wade Road, Basingstoke, Hampshire, England supplied the Gas Generating Kits.

2.4 VECTORS AND HOSTS

Vectors pUC18 and pUC19 as well as host cells JM109 were received from Prof. F. Robb, Department of Microbiology, UCT.

CHAPTER 3 ISOLATION, CHARACTERIZATION AND GROWTH OF C.PYLORI

3.1 ISOLATION AND IDENTIFICATION OF C.PYLORI FROM BIOPSY SPECIMENS

C.pylori isolates were obtained from Dr A.J. Lastovica, Red Cross Hospital, Cape Town. They were isolated and characterized according to the methods described below.

Gastric and duodenal biopsy specimens were obtained from patients who were investigated for gastric ulcers, duodenal ulcers or gastritis at Groote Schuur and Red Cross Children's Hospital. Normally, one specimen was received from each patient, but occasionally, 2 biopsies were taken from different sites in the same patient.

The biopsy specimens were treated as described below:

3.1.1. CULTURE

The biopsy specimen was softened by pressing it against the side of a TBA (tryptose blood agar, Oxoid CM233) plate containing 5% of unlysed horse blood. A fresh sterile swab moistened with TSB (tryptic soy broth) was used to wipe the specimen over the plate once before transferring it to a fresh plate. The applied material was distributed evenly over the plate using a sterile platinum loop. This process

was repeated on a second plate. The biopsy was smeared over the entire surface of a third TBA plate.

C.pylori require a microaerophillic environment (10% CO_2; 5% O_2; 95% humidity) for growth. This was obtained in a CO_2 incubator or in a sealed container with a gas generating system (Gaspak, Oxoid BR56). Plates were incubated at 37°C for 7 days and were examined daily for bacterial growth.

C.pylori are slow growing. Colonies are visible after 4-5 days incubation, they are small, up to 1mm in diameter, circular, colourless and often sparsely distributed. Some isolates, however, show rich, heavy growth. Plates were occasionally covered with fast growing contaminants and it was not always possible to isolate pure cultures of C.pylori since their growth was often inhibited by other organisms. Growth was usually better when plates were incubated with a gaspak system rather than in a CO_2 incubator.

3.1.2. UREASE TEST

C.pylori have been shown to possess very strong urease activity (Goodwin et al 1986), and this characteristic can be used as a diagnostic tool for identifying these organisms. After the inoculation of plates, the biopsy specimens were used to seed Christensen's urea agar slopes, which were incubated in a CO_2 incubator at 37°C overnight.

Many biopsy specimens reacted rapidly on the Christensen's urea agar slope, with a pink colour (positive result) developing within an hour. There was a 50% association between positive urease activity and culture of the organism, but occasionally a negative urease result was associated with a positive but sparse culture of C.pylori. The positive reaction on the urease slope is probably dependant on a threshhold number of organisms being present in the biopsy specimen.

3.1.3. ELECTRON MICROSCOPY

Biopsy material was immersed in electron microscopy fixative (osmium tetroxide) and sent for electron microscopy.

C.pylori isolates were identified under electron microscopy by their characteristic morphology: S-shaped bacilli, with tufts of flagella at one pole.

3.2 CHARACTERIZATION OF C.PYLORI

The media and microaerophillic conditions used for the isolation of organisms from biopsy specimens favoured the growth of C.pylori. Organisms which grew under these conditions were further characterized to identify them as C.pylori.

Colonies were inoculated onto urea agar slopes to test for urease activity. The pink colour usually developed within minutes, but overnight incubation at 37°C was occasionally necessary. Isolates were negatively stained (0,5% phosphotungstic acid) and examined under the electron microscope for their characteristic morphology (M.Emms, personal communication). Furthermore, they were tested for a number of biochemical activities characteristic of C.pylori isolates. The results were compared to those of type strains of C.pylori obtained from the Culture Collection of the University of Goteborg, Sweden and are shown in table 1.

3.3 OPTIMIZATION OF GROWTH CONDITIONS FOR C.PYLORI

3.3.1 GROWTH OF C.PYLORI ON SOLID MEDIA

Growth of C.pylori on the following solid media was compared to establish optimal growth conditions:

1. Tryptose blood agar

2. Boiled blood agar

3. 2% Blood agar

The constituents of each of these media are described in Chapter 2.

C.pylori colonies were visible after 3 days of culture under the previously described incubation conditions on all three of the different types of media, but the colonies were larger on the boiled blood agar. This medium is very rich, however, and plates were often found to be overgrown with other organisms. For this reason, 2% blood agar plates were used.

Since C.pylori is such a slow growing organism, other faster growing organisms frequently contaminated and covered the culture plates. It was essential that all inoculations be done in a laminar flow hood to minimise this problem.

3.3.2 GROWTH OF C.PYLORI IN LIQUID MEDIA

In order to obtain the large numbers of cells required for large-scale DNA preparations, it was necessary to grow C.pylori in liquid media.

Initially, attempts were made to grow C.pylori in para-amino-benzoic acid medium, as other Campylobacter are easily cultured in this medium; inoculated cultures were placed in air-tight containers with a Gaspak in a 37^{o}C incubator for 3 days. No C.pylori grew.

As suggested by Morgan et al (1987), brucella broth (Difco no.3) supplemented with 10% horse serum was used and found to be suitable for growth of C.pylori. It was essential that

all seeding of media be done in a sterile laminar flow hood to minimise contamination. The effect of the size of the inoculum was investigated by picking 5, 10, 20 and 40 isolated colonies off 2% blood agar plates and inoculating each of these into 100ml of medium in a 500ml flask. Cultures were incubated as before, but some flasks were aerated by shaking at 100 rpm. The optical densities of the cultures were measured after 24 and 48 hours of incubation to establish the duration of incubation that resulted in the optimal cell yield.

An inoculum of 40 or more colonies and an incubation time of 48 hours gave the highest optical density. The optical densities of cultures of different sized inocula and different times of incubation are shown in Table 2.

An optical density reading of above 1,5 suggested that the culture medium was contaminated since C.pylori cultures rarely reached such high optical densities. To establish whether culture medium with an optical density of more than 1,5 was contaminated, a loopfull of the culture was spread onto a 2% blood agar plate and incubated at 37^{o}C in an aerobic environment overnight. This usually yielded growth of Staphylococcal organisms, indicating contamination of media at inoculation. Contaminants were also detected by their smell as C.pylori have no odour, and by the colour of the pellet when cells were harvested by centrifugation. A pure culture of C.pylori yields a pinkish-brown pellet; when

the culture medium was contaminated the pellet was partially or wholly creamy-yellow.

C.pylori cultures were identified by a positive urease reaction; a few drops of culture were pippeted onto a Christensen's urea agar slope and the slope was incubated in a 37^{o}C water-bath for 15 minutes.

Table 1* Characteristics of clinical isolates of Campylobacter pyloridis and typed strains of Campylobacter spp.

Isolate	Age(Yr)/sex	growth @ 25°C	37°C	42°C	Hipp-urate	H_2S [1] (FBP)	Cat-alase	Nitrate reduct.[2]	urease[3]	Alkaline phosphatase	aryl sulfatase	DNase[4]	growth on 1% gly	1%bile	3,5% NaCl	TTC[5]	Sensitivity to Nal[6]	Ceph[7]
Clinical:																		
B 1	36/m	-	+	-	-	-	++	-	+++	+++	+++	+++	-	+	-	+++*	R	S
B 4	37/m	-	+	-	-	-	++	-	+++	+++	+++	+++	-	+	-	+++*	R	S
B 6	33/m	-	+	-	-	-	++	-	+++	+++	+++	+++	-	+	-	+++*	R	S
B 11	53/m	-	+	-	-	-	++	-	+++	+++	+++	+++	-	+	-	+++*	R	S
B 15	38/f	-	+	-	-	-	++	-	+++	+++	+++	+++	-	+	-	+++*	R	S
B 16	54/m	-	+	-	-	-	++	-	+++	+++	+++	+++	-	+	-	+++*	R	S
B 18	60/f	-	+	-	-	-	++	-	+++	+++	+++	+++	-	+	-	+++*	R	S
B 21	61/f	-	+	-	-	-	++	-	+++	+++	+++	+++	-	+	-	+++*	R	S
B 24	44/f	-	+	-	-	-	++	-	+++	+++	+++	+++	-	+	-	+++*	R	S
B 25	84/f	-	+	-	-	-	++	-	+++	+++	+++	+++	-	+	-	+++*	R	S
B 33	41/m	-	+	-	-	-	++	-	+++	+++	+++	+++	-	+	-	+++*	R	S
B 38	37/f	-	+	-	-	-	++	-	+++	+++	+++	+++	-	+	-	+++*	R	S
B 45	51/f	-	+	-	-	-	++	-	+++	+++	+++	+++	-	+	-	+++*	R	S
Typed strains:																		
C. pyloridis	CCUG 17135	-	+	-	-	-	++	-	+++	+++	+++	+++	-	+	-	+++*	R	S
	CCUG 15818	-	+	-	-	-	++	-	+++	+++	+++	+++	-	+	-	+++*	R	S
	CCUG 17874	-	+	-	-	-	++	-	+++	+++	+++	+++	-	+	-	+++*	R	S
C. jejuni	ATCC 29428	-	+	+	+	-	+	+	-	++	++	-	+	+	-	++	S	R
C. coli	CCUG 11283	-	+	+	-	-	+	+	-	++	+	++	+	+	-	++	S	R
C. fetus	NCTC 10348	+	+	-	-	-	+	+	-	+	++	-	+	+	-	-	R	S

Symbols: + = positive result, - = negative result, R = resistant, S = sensitive.

1 Iron/bisulphite/pyruvate. Skirrow & Benjamin, J. Hyg. Camb. (1980) 85, 427.

2 Plate test of G.T. Cook, J. Clin. Path. (1950) 3, 359.

3 Christensen's urea agar.

4 toluidine blue DNA agar. Lior et al, Campylobacter II, p. 225 (1985).

5 2,3,5-triphenyltetrazolium chloride, 400 µg/ml. * = metallic sheen to colonies.

6 Nalidixic acid, 0,03 mg discs

7 Cephalothin, 0,03 mg discs

* Table 1 was kindly supplied by AJ Lastovica.

Table 2a:

INOCULUM SIZE (colonies)	OPTICAL DENSITY
5	0,85
10	1,16
20	1,33
40	1,36

Table 2b:

TIME OF INCUBATION (hours) (40 colony inoculum)	OPTICAL DENSITY (Isolate) 1	2	3
24	1,17	0,37	0,33
48	1,79	1,62	1,38

Table 2: Optical densities attained by cultures of C.pylori grown in liquid media with a) various sizes of inocula and b) durations of incubation.

CHAPTER 4 EXTRACTION OF PLASMID DNA FROM C.PYLORI

4.1 SMALL SCALE PREPARATION OF PLASMID DNA

A quick, reliable method of plasmid isolation is required to screen large numbers of recombinant clones. The isolated DNA must be pure enough for restriction enzyme digestion since these enzymes are sensitive to inhibition by impurities. Although mini-preparation methods were developed for this purpose, not all of them yield DNA of a satisfactory purity. Most of the methods have been developed for use with laboratory strains of E.coli; when working with clinical isolates, minor modifications are often needed for the best results. In this work, many modifications of a number of plasmid DNA preparation techniques were investigated to identify which procedures gave the best results.

C.pylori plasmid DNA was extracted by various mini-preparation methods to identify isolates containing plasmid DNA. The DNA obtained by these procedures was also used for mapping with restriction enzymes as difficulties were experienced in obtaining sufficient pure DNA by large scale plasmid preparations.

In the majority of plasmid minipreparation methods, the cells are lysed and the chromosomal and plasmid DNA are

separated by selective denaturation and renaturation. In this work, two principles of selective denaturation and renaturation were used. The first involved SDS lysis of the cells and the alkaline denaturation of chromosomal and plasmid DNA by NaOH at a high pH (pH 12,4), followed by renaturation of plasmid DNA under conditions which resulted in the chromosome forming an insoluble mass which could be separated from the re-annealed plasmid DNA. The second method involved the use of heat to aid alkaline denaturation of DNA released from SDS lysed cells. Due to its supercoiled structure, plasmid DNA is more easily able to renature than chromosomal DNA after denaturation. A number of modifications to each of these methods was investigated and each method was repeated to test its reproducibility.

4.1.1. ALKALINE DENATURATION OF DNA

The method used was that described by Birnboim and Doly (1979). A number of modifications to this method were investigated in an attempt to establish conditions for optimal DNA yield. All steps were carried out in Eppendorf microcentrifuge tubes.

C.pylori were grown as previously described. Cells which had been incubated for various periods of time were used to establish conditions for optimal DNA yield. The best results were obtained with 2-3 day old cultures. Small scale plasmid

preparations were done on pooled colonies from 1, 2 and 3 culture plates. When the colonies from 2 and 3 plates were used, the cells did not lyse completely due to an excess of cells compared to lysis buffer, so all subsequent mini-preps were done using one plate of isolated colonies.

Cells were scraped off the culture plates using a sterile platinum loop and suspended in 180µl of lysis buffer 1 (50mM glucose; 10mM EDTA; 25mM Tris-HCl, pH 8,0). Alternatively, cells were washed off plates with 1,5ml of STE buffer (100mM NaCl; 10mM Tris-HCl, pH7,8; 1mM EDTA) and centrifuged in a microfuge for 1 minute. The cell pellet was resuspended in 180µl of lysis buffer 1.

A spatula tip (3-5mg) of lysozyme powder was added and the tube was vortexed to ensure thorough mixing. The mixture was left at room temperature for 5 minutes to generate spheroplasts before the addition of 400µl of an alkaline/SDS solution (200mM NaOH; 1% SDS, w/v). The tube was vortexed gently and left on ice for 5 minutes, where the solution cleared as the cells lysed.

Chromosomal DNA was precipitated by the addition of 300µl of a high salt solution (3M potassium acetate; 1,8M formic acid) and incubation at $4^{o}C$ for 30 minutes. The chromosomal DNA and cell debris were pelleted by a 15 minute centrifugation in an Eppendorf centrifuge and 750µl of the

supernatant, which contained plasmid DNA, was carefully aspirated. Plasmid DNA was precipitated by the addition of 450µl of isopropanol and pelleted by centrifugation for 5 minutes. The pellet was washed with 70%, cold ethanol and centrifuged for 5 minutes. Thereafter, the pellet was dried under a vacuum (Speed vac concentrator, Serevac).

The DNA pellet was dissolved in 200µl of low salt buffer (10mM Tris-HCl, pH 8,0; 1mM EDTA; 150mM NaCl). Contaminating proteins were denatured and removed by the addition of an equal volume of phenol. After vortexing the tube, 200µl of a chloroform/isoamylalcohol (24:1) solution was added, and the tube was vortexed again. The organic and aqueous phases were separated by centrifugation for 2 minutes and the upper aqueous phase containing the plasmid DNA, was removed.

The plasmid DNA was precipitated by the addition of 500µl of cold, absolute ethanol and by placing the tubes on dry ice for 10 minutes or at -20^{o}C overnight.The DNA was pelleted by centrifugation for 10 minutes at 4^{o}C, washed as previously described and dried under a vacuum. Each pellet was dissolved in 30µl of sterile distilled water.

4.1.1.i) MODIFICATION 1 OF ALKALINE DENATURATION OF DNA

This modification is described by Bradbury et al (1983). The generation of spheroplasts was carried out at 0^{o}C rather

than at room temperature, but the cells were lysed in the same manner as previously described.

After the removal of the chromosomal DNA and the cell debris by centrifugation, 1,0ml of cold 95% ethanol was added to the supernatant, and the plasmid DNA was precipitated at -20°C for 20 minutes and pelleted by centrifugation. The supernatant was discarded and the DNA pellet was resuspended in 100µl of wash buffer (100mM sodium acetate; 50mM MOPS, pH 8,0). This was followed by reprecipitation of the DNA in 200µl of cold ethanol under conditions previously described. The DNA was centrifuged and the 70% ethanol wash and precipitation steps were repeated. The DNA pellet was resuspended in 100µl of a 1:1 H_2O and salt solution (10M lithium chloride; 50mM Tris-HCl, pH 8,0), and the tubes were left on ice for 30 minutes to precipitate proteins and RNA. These were pelleted by centrifugation for 5 minutes and 100µl of the supernatant was removed. The DNA in the supernatant was precipitated with 200µl of cold ethanol. After centrifugation, the DNA pellet was washed in 50µl of wash buffer, reprecipitated and centrifuged. This step was repeated and the pellet was resuspended in 30µl of sterile distilled water.

4.1.1.ii) MODIFICATION 2 OF ALKALINE DENATURATION OF DNA

A second modification of the method was described by Lastovica and Ambrosio (1986) and differs from that of Bradbury et al (1983) in that the pellet is retained after the lithium chloride precipitation, rather than the supernatant. The pellet is resuspended in wash buffer, reprecipitated, centrifuged and resuspended in 30µl of sterile distilled water.

4.1.2. HEAT DENATURATION OF DNA

This method is described by Kado and Liu (1981) and was also modified to increase the DNA yield.

C.pylori were grown as previously described and isolated colonies from half of a culture plate were used for each experiment.

4.1.2.i) MODIFICATION 1 OF HEAT DENATURATION OF DNA

Cells were scraped off plates and suspended thoroughly in 150µl of E buffer (40mM Tris-acetate, pH 8,0; 1mM EDTA). Lysis was accomplished by the addition of 300µl of lysing solution 2 (3% SDS; 50mM Tris-HCl, pH 12,6; 320Mm NaOH) and by incubation at 56^{o}C for 1 hour. The lysate was extracted with phenol-chloroform and the DNA was precipitated with a

1/10 volume of 4M LiCl and 2,5 volumes of absolute ethanol at -20°C overnight. After centrifugation, the pellet was washed with 70% ethanol, dried and dissolved in 30µl of sterile distilled water.

4.1.2.ii) MODIFICATION 2 OF HEAT DENATURATION OF DNA

A second modification of this method is described by Ambrosio and Lastovica (1983). Cells were suspended in 1ml of buffer (50mM Tris-HCl, pH 8,0; 2mM EDTA). The lysing solution 2 described above was added only after the cells had been treated with proteinase K (100µg/ml). Cells were lysed by incubation at 37°C for 15 minutes followed by incubation at 70°C for 30 minutes. The lysate was extracted with phenol-chloroform and centrifuged to separate phases. The clear aqueous phase was removed and aliquots (80µl) were taken for electrophoresis.

4.1.2.iii) MODIFICATION 3 OF HEAT DENATURATION OF DNA

A third modification of this method was described by Willimzig (1985). Cells were scraped off one culture plate and suspended in 100µl of TELT buffer (50mM Tris-HCl, pH7,5; 62,5mM EDTA; 0,4% Triton X-100; 2,5M LiCl). A spatula tip (3-5mg) of lysozyme powder was added, mixed and the solution was placed in a boiling water bath for 1 minute. The mixture was allowed to cool on ice for 5 minutes, whereafter it was

centrifuged for 8 minutes at room temperature. The supernatant was retained and the DNA precipitated with ethanol at -70°C for 10 minutes. After centrifugation the DNA pellet was washed with 70% ethanol, dried and dissolved in 30µl of sterile, distilled water.

4.1.3 COMPARISON OF VARIOUS PLASMID MINIPREPARATION METHODS

Table 3 summarises and compares the results obtained using the various small scale plasmid DNA preparation methods. The unmodified method of Birnboim and Doly gave the most consistent results and yielded the highest concentration of DNA. Washing the cells off plates with STE made no visible difference to the quantity or quality of DNA recovered, so this step was omitted for later experiments.

A contradiction exists in two of the modifications described for the Birnboim and Doly plasmid minipreparation method. Modification 4.1.1.i described by Bradbury et al (1983) uses a 5M lithium chloride solution to precipitate proteins and RNA, while modification 4.1.1.ii described by Lastovica and Ambrosio (1986) uses a 5M lithium chloride solution to precipitate DNA. This contradiction was investigated by retaining both the pellet and the supernatant after precipitation with lithium chloride and centrifugation, and further puryifying both for electrophoresis. After electrophoresis, more DNA was shown to be present in the

supernatant, but there was a detectable amount of DNA in the pellet. A low concentration of lithium chloride (0,4M), is used as a routine method to precipitate DNA while according to Willimzig (1985), a 2,5M solution of lithium chloride can be used to remove ribosomal RNA and proteins. The results obtained here using a lithium chloride concentration of 2,5M, showed that DNA was precipitated together with proteins and RNA, indicating that this technique is not as selective as is claimed.

The method of Kado and Lui (1981) and the modification thereof gave low yields of impure DNA and were thus unsuitable.

The lithium-chloride boiling method gave higher yields of DNA than the previous two methods, but there was chromosomal contamination of the DNA which masked the plasmid profiles in some isolates. Despite the high yields, the purity of the DNA was poor and this method was not used routinely.

Table 3 : A comparison of the results obtained by various small scale plasmid preparation methods.

	YIELD	CHROMOSOMAL CONTAMINATION	TIME TAKEN	REPRODUCI-BILITY
ALKALINE DENATURATION				
Birnboim and Doly	Good	Very Little	3,5 hours	Fair
Modification 1	Fair	Moderate	5 hours	Poor
Modification 2	Poor	Moderate	5 hours	Poor
HEAT DENATURATION				
Modification 1	Poor	High	25 or 3 hours	Fair
Modification 2	Poor	High	3 hours	Poor
Modification 3	Good	High	1,5 hours	Fair

4.2. LARGE-SCALE PREPARATION OF PLASMID DNA

The enhanced growth of C.pylori in liquid media allowed large numbers of cells to be cultured for the preparation of plasmid DNA on a large scale. High concentrations of pure DNA are required for mapping with restriction enzymes; cloning; transformation; hybridization or sequencing.

The method used was similar to that described by Greenaway and Dale (1983). Morgan et al (1987) showed that maximum dispersion of gases throughout the liquid medium was essential for the growth of C.pylori. To achieve this, 100ml of inoculated culture medium was measured into a 500ml flask which was placed inside an air tight container together with a CO_2 generating system (Gaspak). The cultures were aerated at 37^{o}C on an orbital shaker at 100rpm for 48-60 hours.

Cultures were examined for purity as set out in section 3.2. If the cultures were uncontaminated, the cells were harvested by centrifugation in Corex tubes (Corning) in a J2-21 centrifuge (Beckman) at 4000 rpm in a JA20 rotor at 4^{o}C. The supernatant was discarded and the cells were resuspended in 3ml of lysis buffer (50mM Tris-HCl, pH8,0; and 25% sucrose, w/v). Spheroplasts were generated by the addition of dry lysozyme powder (8-10mg), and the intermittent swirling of the tubes which were kept on ice for 5 minutes. Thereafter, 3ml of 0,25M EDTA , pH8,0, was

added and the tubes were again swirled on ice for 5 minutes. The cells were lysed by the rapid addition of 4ml of a Triton/DOC or Brij/DOC solution (1% Triton or Brij; 4% sodium deoxycholate in 10mMM Tris, pH 8,0; 1mM EDTA).The solution was mixed with the cells by drawing them up into and expelling them from a 10ml plastic pipette three times. After the tubes had been kept on ice for 30 minutes, Sarkosyl (0,5%, v/v) was added and the cells were kept on ice for a further 20 minutes to ensure complete cell lysis. The addition of 1% SDS also lysed the cells, but was shown to alter the density of the solution and thus the separation of the chromosomal and plasmid DNA bands after ultracentrifugation was impaired.

Cell debris and chromosomal DNA were pelleted by a 2 hour centrifugation at 4^{o}C and 8000 rpm using the centrifuge and rotor described earlier. The cell lysate was carefully removed and its volume was measured; if it was less than 10ml, the volume was made up to 10ml with the Triton/DOC solution. Solid CsCl was added (0,95g/ml) and the tubes were gently rocked to dissolve the CsCl. When the CsCl was completely dissolved, 200µl of a 10mg/ml ethidium bromide solution was added and thoroughly mixed into the solution. The CsCl solution was centrifuged in 5ml polyallomer tubes in a Vti65.2 rotor in an ultracentrifuge (Beckman L7) at 50000 rpm for 20 hours. After centrifugation the tubes were

illuminated with ultraviolet light to visualize the DNA bands.

Theoretically, a single, well circumscribed plasmid band would be found below the chromosomal band, but this was not the case for C.pylori. Occasionally, it was possible to distinguish 2 bands below the chromosomal band. This may have been the result of the cells being harvested before they reached the stationary phase of their growth, so that the plasmids were still replicating and not yet fully supercoiled at the time of harvest. Normally, one faint, broad band was noted. When 2 bands were present, they were harvested separately. The harvested DNA was extracted 3 times with an equal volume of isoamylalcohol to extract the ethidium bromide. This was followed by dialysis of the DNA against 3 litres of STE at 4^{o}C overnight to remove the CsCl; the buffer was changed after 4 hours. The DNA was recovered from the dialysis tubes and precipitated with ethanol at -20^{o}C overnight. If the volume recovered after dialysis was less than 1ml, the tubes were centrifuged at 12000rpm in an Eppendorf centrifuge at 4^{o}C. For larger volumes, the tubes were centrifuged in a J2-21 centrifuge (Beckman)at 8000rpm in a JA20 rotor for 1 hour. The DNA pellet was washed with 70% ethanol and dried. The DNA was redissolved in 200µl of sterile, distilled water.

4.2.1 MODIFICATIONS OF THE METHOD OF GREENAWAY AND DALE

Modifications were made to the above method in an attempt to obtain greater yields of DNA. It was noticed that when removing the dialysed solutions from the dialysis tubes, much of the liquid was left on the inner surface of the tubes. To avoid this loss of DNA, the dialysis step was omitted, and the CsCl solution was diluted by the addition of 2 volumes of distilled water; the DNA was precipitated with ethanol, washed with 70% ethanol, dried under a vacuum and redissolved as before. In another modification, the chromosomal band was harvested together with the plasmid band. The volume of the recovered solution was measured and made up to 10ml with Triton/DOC. CsCl and ethidium bromide were added to a final concentration as before. Ultracentrifugation was repeated to ensure separation of the plasmid DNA from the chromosomal DNA and this time, only the plasmid band was harvested.

These modifications did not improve the yield or purity of the DNA and were not used in later experiments.

CHAPTER 5 ANALYSIS OF PLASMID DNA

5.1 AGAROSE GEL ELECTROPHORESIS

Electrophoresis of DNA through agarose gels can be used to separate, identify, purify and establish the size of DNA fragments. The DNA is loaded into pre-formed slots in the agarose gel which is immersed in an electrophoresis buffer. At neutral pH values, the phosphate backbone of the DNA is negatively charged and under the influence of an electric current, the DNA will migrate towards the anode. After electrophoresis, the gel can be stained with ethidium bromide, a dye which intercalates between the bases of the DNA and flouresces in the presence of ultraviolet light. By transilluminating the gel with ultraviolet light, the DNA fragments and can be visualised. The rate of migration of DNA through the agarose is dependant on a number of factors which are discussed below.

5.1.1 FACTORS AFFECTING THE RATE OF MIGRATION OF DNA IN AGAROSE GELS

1) Molecular size of the DNA

The electrophoretic mobility of the DNA is related to the $\log_{10}$ of the molecular weight of the DNA. This characteristic is used to determine the molecular weight of fragments of DNA by comparing their mobilities

with those of fragments of known molecular weight (see Appendix 3 for a typical standard curve used to calculate the sizes of fragments). DNA is believed to migrate through the agarose in an end-on position (Maniatis 1982) and larger molecules take longer to weave a path around the agarose beads in the gel than smaller ones. For a given time of electrophoresis, therefore, smaller molecules will have migrated further from the origin than larger ones in an agarose gel.

2) The agarose concentration

The rate of migration of a DNA fragment of a given size decreases as the gel concentration increases. The gel concentration is chosen to give optimal separation of fragments of a particular size. Double stranded DNA fragments of 70 bp and 800kb can be analyzed on 3% and 0,1% agarose gels respectively, (Sealy and Southern 1982). The 3% gels, however, are brittle and the 0,1% gels are flimsy; this makes them difficult to handle and a concentration somewhere between these two extremes is more practical for general use. For this work, fragments of 0,5kb to 40kb were separated on 0,8% agarose gels.

3) Conformation of the DNA

Plasmid DNA can exist in 3 different conformations:

i) covalently closed circular DNA

ii) open circular DNA

iii) linear DNA.

Covalently closed circular DNA is tightly supercoiled and has a compact structure. This allows it to migrate through agarose gels more quickly than linear and open circular DNA, whose more diffuse structures retard their movement between the agarose beads.

4) The applied current

The strength of the applied current is chosen on the basis of the sizes of fragments to be separated, the degree of separation required and the time available (Sealy and Southern 1982). For large fragments (>5kb), maximum separation is achieved by electrophoresis at low voltages for long periods of time (overnight); small fragments can be separated adequately at high voltages (70V) for short periods of time. When separating DNA fragments with a wide range of sizes, the voltage is chosen so as to achieve maximum separation of fragments, but with minimum diffusion of small bands, which reduces their sharpness.

5.1.2 METHODOLOGY

Agarose beads were suspended in TAE buffer (0,04M Tris-acetate; 0,01M EDTA) and dissolved in a microwave oven.The solution was allowed to cool to 56^{o}C before it was poured into a casting tray with the slot former already in place. When the agarose was set the slot-former was removed and the

gel was immersed fully in TAE buffer in an electrophoresis tank. The DNA samples were mixed with an appropriate amount of 6x loading buffer (0,25% bromphenol blue; 0,25% xylene cyanol; and 15% Ficoll, type400) and pipetted carefully into the slots. The purpose of the loading buffer was: to increase the density of the DNA so that it did not float out of the wells; and to allow an estimation of the distance travelled by the DNA during electrophoresis by monitoring the distance travelled by the indicator dyes present in the loading buffer.

DNA loaded onto large gels (22cmx12cm) was electrophoresed overnight at 18-30V, while that on small gels (checking gels of 10cmx7,5cm) was electrophoresed for 60-90 minutes at 65-75V. When fragment sizes had to be accurately determined, the gel was stained after electrophoresis in distilled water containing 30µl of a 10mg/ml ethidium bromide solution per litre of water for 30 minutes. For checking gels, one drop of a 100µg/ml solution of ethidium bromide was added to 50ml of molten agarose before electrophoresis. As stated earlier, ethidium bromide intercalates between the base pairs of the DNA; this results in a slight alteration in the structure of the DNA (particularly supercoiled DNA) and therefore influences the rate of migration of DNA through agarose gels.

5.2 ANALYSIS OF PLASMID PROFILES

5.2.1 GENERAL FEATURES OF C.PYLORI PLASMID PROFILES

In this study, 48 C.pylori isolates were examined for the presence of plasmids. Twenty six (54%) of them were shown to contain plasmids with a wide variety of electrophoretic migration patterns (see Figure 3). When compared with covalently closed circular plasmids of known molecular weight, the plasmid bands were shown to be comparable in size with those of 3.4 to greater than 137kb. Based on the varying intensities of the plasmid bands, the plasmid copy number appeared variable both between isolates and for different plasmids from the same isolate. The majority of isolates had unique plasmid profiles, but in 5 isolates the profile was identical. The plasmid profiles of three of these isolates can be seen in Figure 3, lanes A, B and C. The ladder of plasmid bands seen in lanes B and I of Figure 3 represent plasmids with varying degrees of supercoiling and are discussed at the end of section 5.2.2. When separate biopsies were taken from different areas from the same patient, the organisms cultured from each of these biopsies were compared by their plasmid profiles. For one patient, the two organisms cultured from 2 different biopsies showed identical plasmid profiles (see lanes E and F, Figure 3), while for another patient, the two isolates had different plasmid profiles. This suggested that the latter patient may

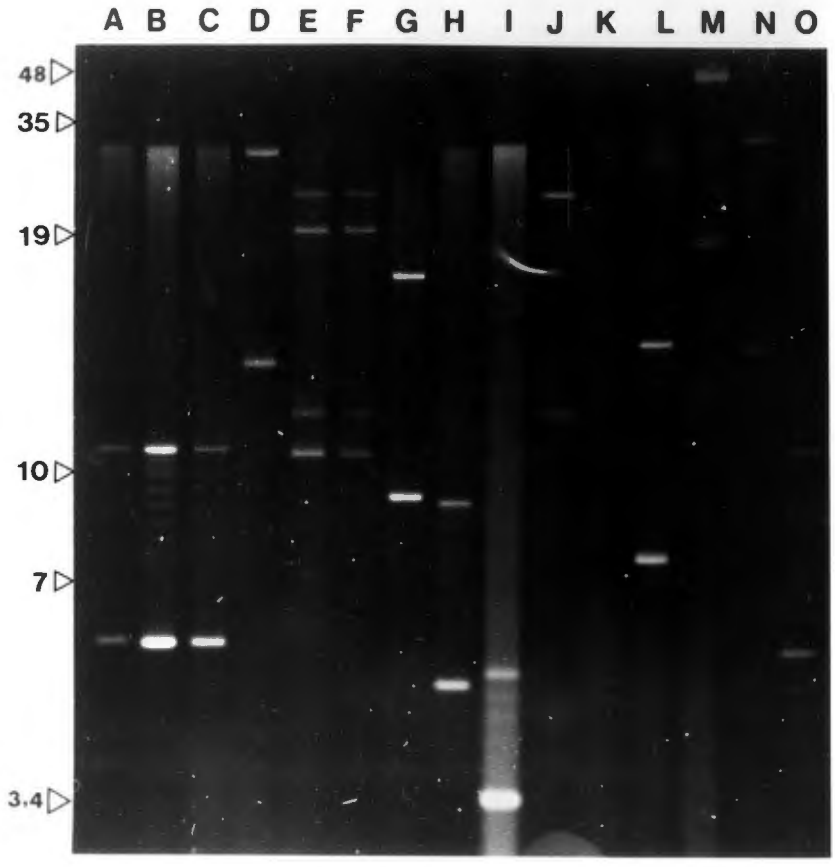


Figure 3: A demonstration of the variety of plasmid profiles found in C.pylori. Lanes A, B and C show three isolates with a common plasmid profile, while lanes E and F demonstrate the plasmid profiles of 2 isolates taken from the body and antrum of the stomach of the same patient.

Plasmid sizes are given in kilobase pairs. For an explanation of the ladder visible in lane B, see text page 73.

have had a mixed infection of C.pylori. The plasmid yield was often poor, with bands being barely visible after electrophoresis. Many isolates showed more than one plasmid band, representing a number of different plasmids or different conformations of the same plasmid.

5.2.2 COMMON PLASMIDS IN C.PYLORI

Three (B1, B2 and B3) of the the 5 isolates with a common plasmid profile were investigated further to determine whether they shared identical plasmids and whether they shared an identifiable common characteristic which could be attributed to any of these common plasmids. In these isolates, 3 plasmid bands were present (see Figure 4). The relative positions of these three plasmids and the fact that not all of the three were present in some preparations suggested that they may be alternative conformations of the same plasmid (such as covalently closed circular, nicked open circular, or linear, labelled c, a and b respectively in figure 4). When the plasmids were gel purified after agarose gel electrophoresis and re-electrophoresed, they frequently changed their mobilities: the fastest migrating band (c) showed mobilities identical to the two slower migrating bands; the middle band (b) showed its original migration pattern, while the slowest migrating band (a) showed its original mobility as well as that of the middle plasmid (b). The altered electrophoretic migration patterns

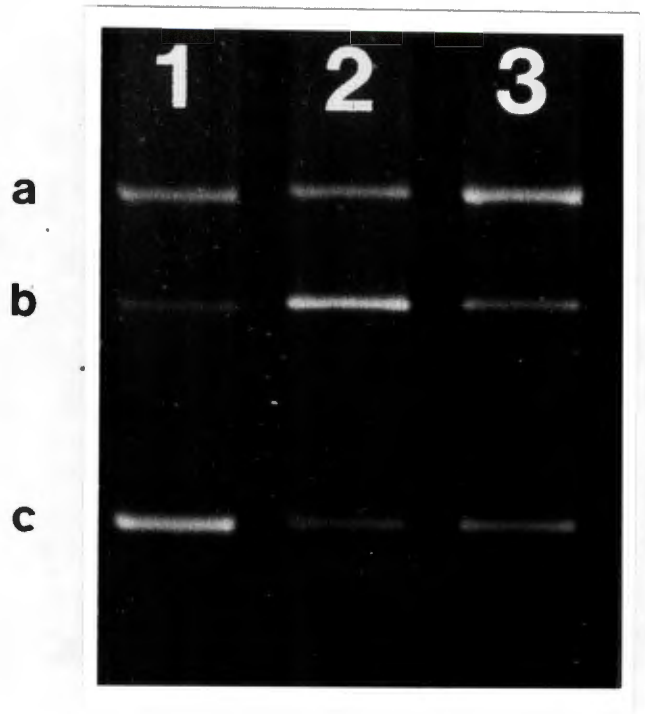


Figure 4: Five isolates showed this plasmid profile and the 3 shown here were studied further. Open circular DNA is labelled (a), linear DNA is labelled (b) and covalently closed circular DNA is labelled (c).

could be due to damage to the DNA during gel purification which results in the generation of alternative conformations of the plasmids. The exact nature of the changed migration patterns supports this theory: the tightly supercoiled plasmid (c) could be nicked in one or both strands to generate open circular DNA (a), or linear DNA (b), respectively; the open circular DNA (a) would be converted into linear DNA (b) if the remaining intact strand was sheared; and damage to the linear DNA (b) would not change its conformation and this DNA would thus retain its original electrophoretic migration pattern.

The gel purified plasmids were digested with HindIII to see if this enzyme recognised the same sites on these 3 plasmids. Results obtained were difficult to interpret as the fragments were extremely faint after agarose gel electrophoresis and partial digestion had occured. It was possible to distinguish the restriction patterns of the slowest and fastest migrating bands (a and c respectively in figure 4) and these were identical, indicating that they were the same plasmid. No restriction pattern could be visualized for the middle band however, so it was not possible to compare this plasmid with the other two.

Further indications that these 3 bands represent alternative conformations of the same plasmid came from large scale plasmid preparations. In most of these preparations, only 2

bands were visible. These were the two slowest migrating of the three plasmid and the possibility that the slowest migrating plasmid could have been lost through continuous passaging of the organisms had to be excluded. When small scale DNA preparations were done on cells which had shown only two plasmid bands for large scale plasmid preparations, all 3 plasmid bands were present in these isolates. This indicated that the fastest migrating plasmid (c) had not been lost from the isolates, but rather was not being isolated by the techniques described for large scale plasmid preparations. The techniques employed in this method involved many manipulations of the DNA which may have damaged it and destroyed its supercoiled structure. Loss of this conformation would alter the mobility of the DNA during agarose gel electrophoresis.

More evidence for the hypothesis that these three bands represent alternative conformations of the same plasmid comes from the results of restriction enzyme digests of total plasmid DNA. Although most enzymes appeared not to digest the DNA, EcoRI either partially digested the plasmids or resulted in an alteration in the intensity of the three plasmid bands after restriction enzyme digestion. The fastest migrating band decreased in intensity while either of the other two bands, particularly the intermediate band, increased in intensity. The decrease in intensity of the fastest migrating band could be the result of a decrease in

the amount of supercoiled DNA due to its being nicked or digested. Non-specific nicking or digestion of the DNA may occur when the enzyme reaction is allowed to continue for a longer period of time than that recommended by the supplier (as was done for many of the digests). The nicking or linearising of supercoiled DNA would then be accompanied by an increase in the intensity of the slower migrating bands, as was the case here, if these represented nicked and linear DNA.

In some plasmid preparations, a faint ladder of plasmid bands of increasing size was vizualised between the fastest and intermediate migrating bands (see Figure 3, lane B). Since these bands were only present in some preparations, it appeared that some conditions of growth which were difficult to control favoured their formation. The nature of these bands suggests that a change had occured in the linking number of the plasmids ie. they were progressively less supercoiled (Warren and Green, 1985).

5.3 RESTRICTION ENZYME DIGESTION OF DNA

Restriction endonucleases recognise specific sequences of bases in double-stranded DNA and cleave the phosphodiester backbone of the DNA. The enzymes that were used in this study are class II restriction enzymes; their recognition sites are 4 or 6 nucleotide, palindromic sequences and restriction occurs within or just outside these sites. These

enzymes make many molecular studies and manipulations possible. Many of them make staggered cuts in the DNA, which result in cohesive ends that can be used to link foreign fragments of DNA which have complementary ends, and thus produce recombinant molecules. The mapping of DNA fragments is also made possible through the use of restriction enzymes: the DNA is digested with selected enzymes and the fragments can be separated by electrophoresis; the number and size of fragments produced by an enzyme can be determined and used to construct a restriction enzyme map of the DNA.

In this work, restriction enzyme digests were initially carried out under the conditions specified by the suppliers. When partial digestion occurred, the time of incubation was increased to 18 hours and a 10-100 fold excess to the recommended amount of enzyme was used to obtain complete digestion. Care was taken to ensure that the volume of the added enzyme mixture never exceeded 10% of the total volume of the reaction mixture, as the glycerol in the enzyme buffer is known to inhibit enzyme activity at concentrations of greater than 10%.

If possible, when digests with 2 different enzymes were performed, a restriction buffer was chosen which is suitable for both enzymes. If the enzymes required markedly different salt conditions for their action, the digests were carried

out in succession with the enzyme requiring the lowest salt concentration being used first. After digestion, the reactions were terminated, the DNA was precipitated with ethanol and resuspended in the buffer required for the second enzyme; or the reaction mixture was heated for 10-15 minutes to a temperature (65-80^{o}C) which inactivated the enzyme and an appropriate amount of salt was added to increase the salt concentration to that required by the second enzyme.

After the last digest, the appropriate amount of 10x stop buffer (50% v/v glycerol; 100mM EDTA, pH 8,0; 1% w/v SDS; 0,1% w/v bromophenol blue and 0,1% cyanol) was added to terminate the reaction. The digested DNA was electrophoresed on the same gel as fragments of known molecular weight (usually lambda DNA digested with Hind III). The distances migrated by these standards were plotted against the logarithm of their molecular weights to establish a calibration curve which could be used to estimate the size of the restriction fragments (see appendix 3).

5.4 RESTRICTION ENZYME MAPS OF C.PYLORI PLASMID DNA

Plasmid DNA from three (B1, B2, B3) of the 5 isolates with identical plasmid profiles was analyzed by restriction enzyme digestion to establish whether the plasmids were identical in these isolates. The two isolates with this plasmid profile that were not included in this study were

cultured from biopsy specimens taken towards the end of the study.

5.4.1 RESTRICTION PATTERNS OF C.PYLORI

When digests were done on small scale DNA preparations from isolates B1, B2 and B3 with a variety of enzymes, namely, HindIII, EcoRI, PstI, HaeII and BamHI, none of the enzymes appeared to digest the DNA. Since impurities present in DNA are known to inhibit the activity of restriction enzymes, it was thought that the DNA prepared by the minipreparation method contained impurities which inhibited digestion. In an attempt to purify the DNA further, it was passed through a spun column containing Sephadex G50 in water in a 1ml Tuberculin syringe. The column was prepared as described in section 9.3.1. The DNA was collected and digested under similar conditions as described previously. There was still no digestion.

DNA was also prepared by the small scale method from cells grown in liquid media rather than on solid media. When this DNA was compared by agarose gel electrophoresis with undigested DNA from cells grown on solid media, there appeared to be more background material present. However, restriction enzyme digestion on this DNA was slightly more effective. This improved digestion was monitored by a change in the relative intensities of plasmid bands as described in

section 5.2.2. DNA isolated from cells grown on solid media was more resistant to restriction enzyme digestion than that from cells grown in liquid media.

DNA prepared by CsCl/EtBr ultracentrifugation is relatively pure and suitable for restriction enzyme digestion, transformation and hybridization. Thus, for restriction enzyme mapping , C.pylori plasmid DNA was prepared by this method.

Digestion of the DNA under conditions specified by the suppliers still failed to produce results which would allow the construction of an accurate restriction enzyme map of the plasmids. Two enzymes, EcoRI and HindIII, usually cut the DNA partially. The number and size of fragments generated by digestion with these enzymes varied for consecutive digest reactions, although some of the fragments produced were characteristic for a particular enzyme. Restriction enzyme HindIII consistently produced two small fragments of 1,2kb and 1,3 kb (marked in lane 3 of Figure 5) and EcoRI produced a .6,2kb fragment in a number of digests.

This 6,2kb fragment produced by digestion with EcoRI was assumed to represent linearised plasmid, as it represents the approximate sum of all the smaller fragments generated in HindIII digests. It appeared that not all the sites for a particular restriction enzyme were being recognised/cleaved in every digest. For each digest, a series of bands

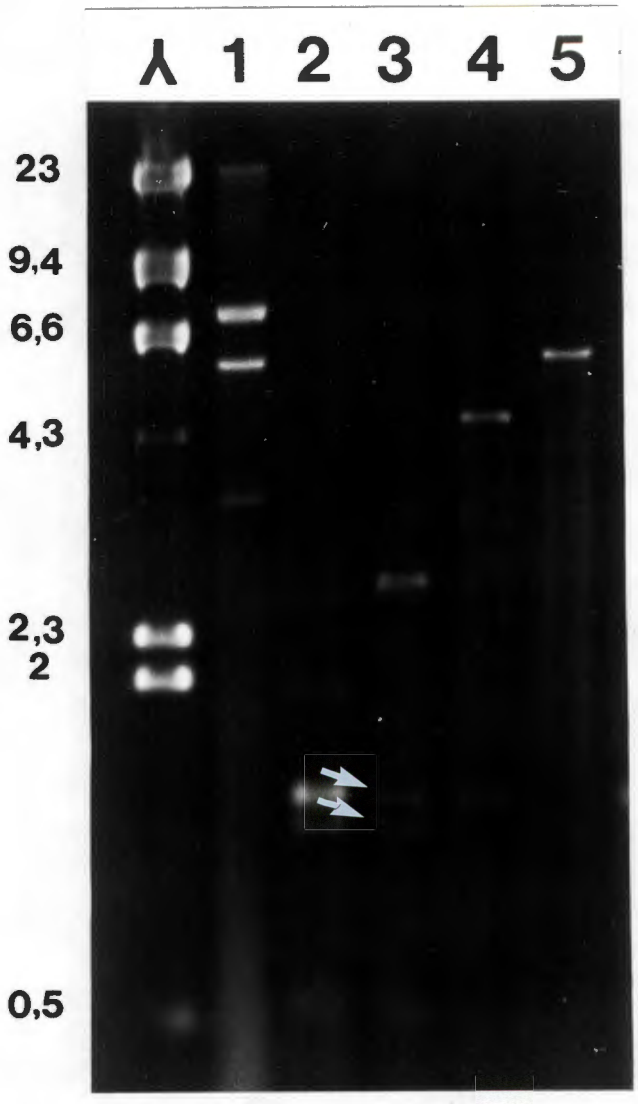


Figure 5: Restriction fragment patterns of C.pylori DNA with fragment sizes given in kilobase pairs. Lane 1 shows undigested plasmid DNA from isolate B2. Lane 5 shows the plasmid recovered off a CsCl/EtBr gradient and used for digestion. Lane 2- double digest with EcoRI and HindIII; lanes 4 and 3: EcoRI and HindIII digests respectively.

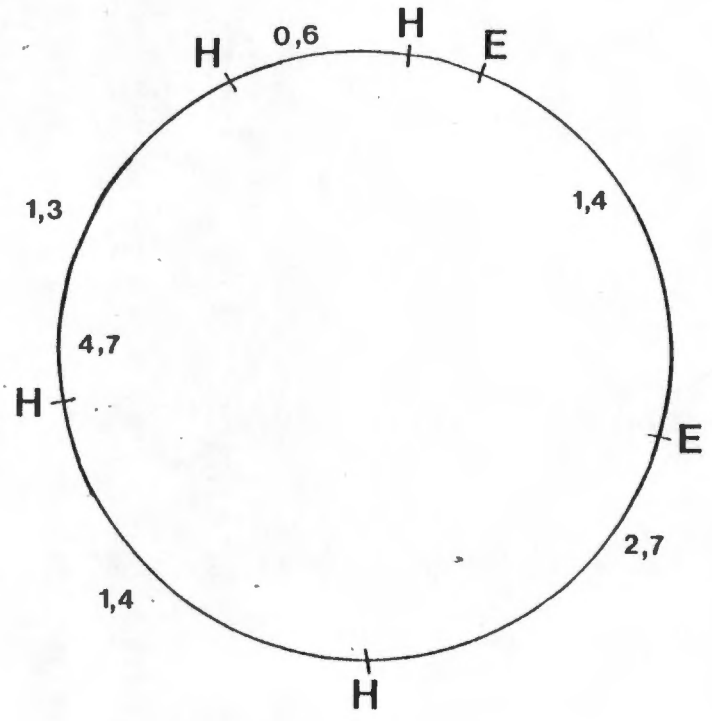


Figure 5A: A provisional restriction enzyme map of an approximately 6,2 kb plasmid of C.pylori.
Figures outside the circle represent the sizes, in kilobase pairs, of Hind III fragments, while those inside represent EcoRI fragments.
E: EcoRI restriction sites
H: Hind III restriction sites

containing various combinations of fragments was visible. As a result of this, some restriction fragments were represented more than once in the restriction pattern obtained, (on their own or combined with other fragments generated by partial digestion) so it was difficult to calculate the total size of the plasmids. In an effort to get complete digestion of the DNA by the enzymes, the digestion time was increased from 3 hours to 18 hours and a 100 fold excess of enzyme to that suggested by the suppliers was used. This still did not result in complete digestion with restriction enzymes EcoRI, HindIII, PstI, BglII, HpaI, XbaI, AvaI and NcoI.

5.4.2 REASONS FOR THE LACK OF DIGESTION OF C.PYLORI PLASMIDS

The failure of so many enzymes to digest C.pylori DNA suggested that either the methods of preparation of the DNA failed to yield sufficiently pure DNA for restriction enzyme activity; or that there was some property of C.pylori DNA which made it refractory to digestion: or that no sites were present for the enzymes tested. Since attempts at purification of the DNA failed to improve digestion, the second hypothesis was investigated. To test this, DNA with a known restriction pattern for a particular enzyme (lambda cut with HindIII or EcoRI) was mixed with C.pylori DNA before digestion by this enzyme. Digestion was performed as

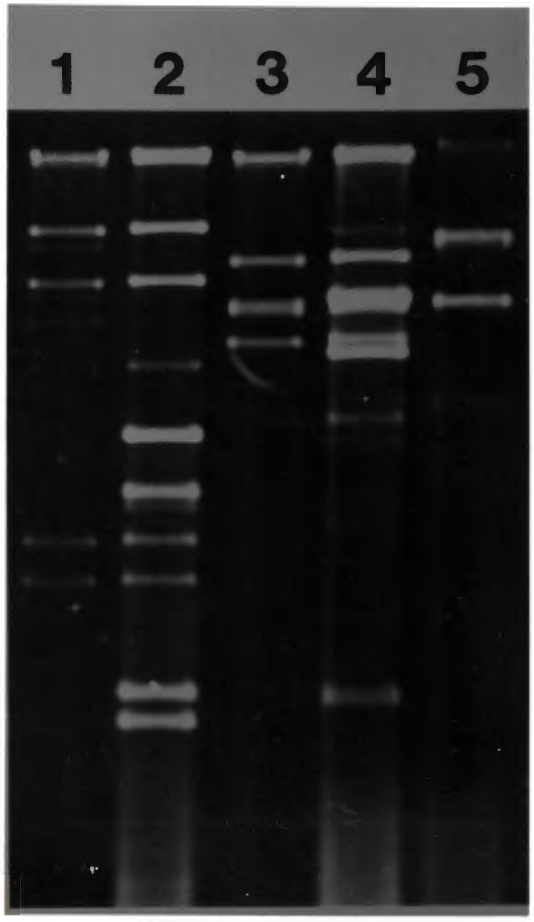


Figure 6: Demonstration that C.pylori DNA is refractory to digestion. Lane 1: lambda DNA digested with HindIII;lane 2: lambda DNA and C.pylori DNA digested simultaneously with HindIII; lane 3: lambda DNA digested with EcoRI; lane 4: lambda DNA and C.pylori DNA digested simultaneously with EcoRI; lane 5: C.pylori DNA undigested.

instructed by the suppliers. A pure sample of lambda DNA was also digested with this enzyme. After digestion, the DNA was electrophoresed and the restriction enzyme patterns were compared. All the fragments generated by HindIII and EcoRI digestion of pure lambda DNA were present in the tube containing both digested lambda and C.pylori DNA. The tube containing the DNA mixture also had a series of fragments representing a partial digest of C.pylori DNA (see Figure 6, lanes 2 and 4). These results implied that no inhibitor was present in the C.pylori DNA, but rather, it was a property of C.pylori DNA which prevented digestion by restriction enzymes. If inhibitors had prevented the digestion C.pylori DNA, the digestion of lambda DNA in the solution would also have been inhibited.

One property of DNA known to affect the activity of many restriction enzymes is if the DNA is methylated. Many enzymes do not cleave their restriction sites if an adenine or cytosine residue within the sequence that they normally recognise is methylated. Isoschizomers are enzymes which recognise the same sequence of bases, and may differ in their ability to restrict methylated DNA. Two such enzymes are Sau3A and MboI. Both recognise the sequence GATC, but only Sau3A digests methylated DNA. C.pylori plasmid and chromosomal DNA was digested by these enzymes and the results are shown in Figure 7. Compared with the undigested DNA shown in lane 1, no digestion occured with MboI (lane

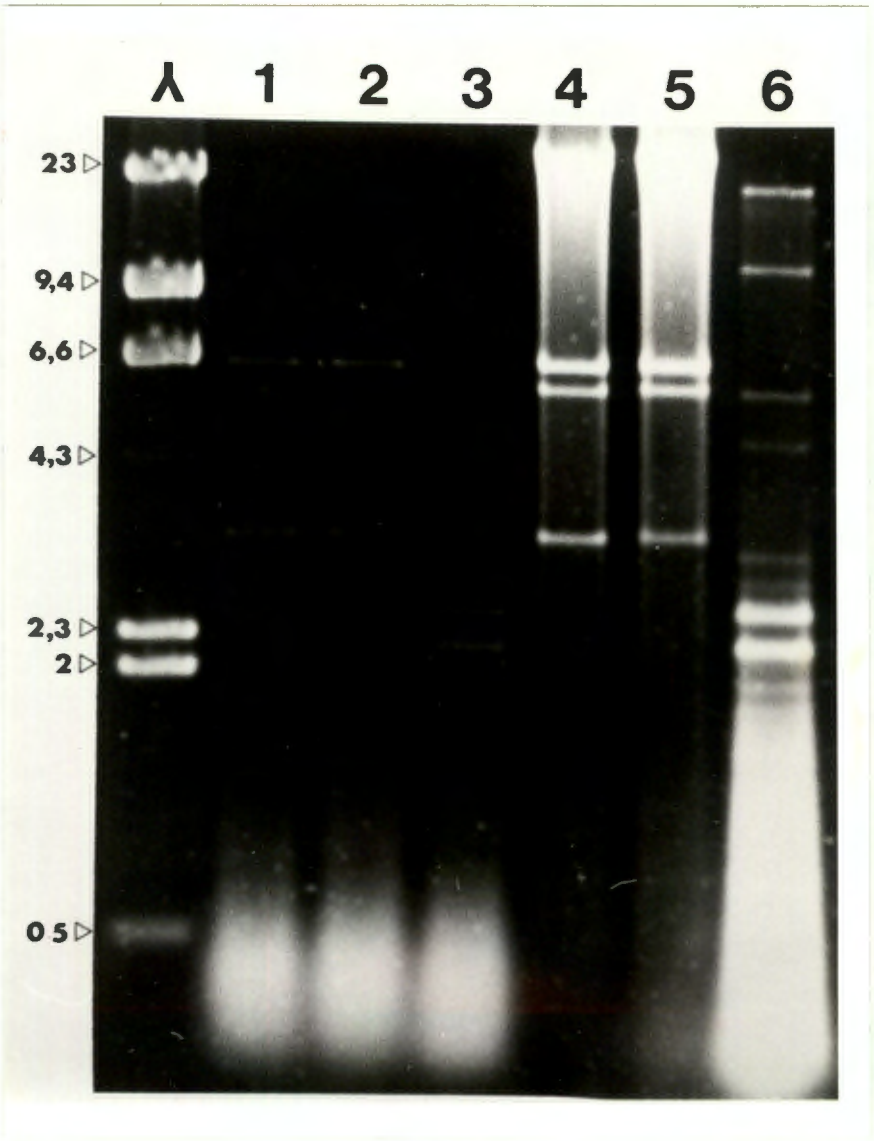


Figure 7: Demonstration that C.pylori DNA is methylated. Lanes 1, 2 and 3 show C.pylori plasmid DNA undigested, digested with MboI and Sau3A respectively. Lanes 4, 5 and 6 repesent C.pylori chromosomal DNA undigested, digested with MboI and Sau3A respectively. Fragment sizes are given in kilobase pairs.

2), but all 3 bands were digested with Sau3A (lane 3). Similarly, chromosomal DNA was digested by Sau3A (lane 6), but not by MboI (lane5), when compared with undigested chromosomal DNA (lane 4).This suggested that the DNA was methylated. Identical results were obtained when the experiment was repeated with fresh MboI enzyme to confirm that the lack of restriction was not due to decreased activity of the MboI enzyme. In an attempt to establish the methylation pattern of C.pylori DNA, the DNA was digested with a series of enzymes known to be specifically sensitive to either adenine methylation, cytosine methylation or methylation of both residues (see Table 3). These results failed to establish the particular methylation pattern of C.pylori DNA as almost all the enzymes used, regardless of their particular methylation sensitivity, failed to digest C.pylori DNA.

The failure of so many enzymes to digest C.pylori DNA and the difficulties encountered in trying to identify a specific methylation pattern which may be responsible for preventing restriction enzyme digestion, suggested that there was something unusual about the common plasmids of C.pylori. This theory was substantiated by the restriction enzyme pattern obtained by digestion with Sau3A. This enzyme recognises a 4 base pair sequence of nucleotides. Statistically, one would expect to find this sequence once in every 4^4 (256) nucleotides. If the plasmid in question is

6,2kb large, one would expect it to be cut into approximately 24 (6200/256) fragments, each about 256bp long. However, digestion with Sau3A resulted in the production of 2 fragments, 2,4 and 2,2 kb each. These fragments would account for 4,6kb of the 6,2kb plasmid. Presumably, the other 1,5kb of the plasmid are digested as one would expect, to yield a series of small fragments of approximately 256bp in length, which would appear as a smear at the bottom of the gel. Why then, does Sau3A fail to digest C.pylori DNA as frequently as one would expect and why does it yield the specific restriction pattern that it does for C.pylori DNA? A possible explanation for this phenomenon is that the plasmid contains a repetitive element. If a particular sequence of bases is repeated and this sequence does not contain any restriction sites, then no restriction will occur within that repetitive element. The 2,2 and 2,4kb fragments generated may be two such sequences consisting of a series of repeated base pairs which do not contain any restriction sites for the enzymes used.

Table 3: Enzyme sensitivities to adenine and cytidine methylation and their ability to digest C.pylori DNA. A- adenine; C- cytidine.

ENZYME	SENSITIVITY TO A METHYLATION	SENSITIVITY TO C METHYLATION	DIGESTS C.PYLORI DNA
HindIII	+	-	Partially
EcoRI	+	+	Partially
KpnI	-	-	No
Sau3A	-	+	Yes
SphI	-	-	No
RsaI	-	-	No
MboI	+	-	No

CHAPTER 6 EXTRACTION OF CHROMOSOMAL DNA FROM C. PYLORI

Up to this point, all DNA studies had been carried out on the plasmid DNA of C.pylori isolates. Five isolates had shown identical plasmid profiles and limited restriction enzyme analysis on the plasmid DNA from three of these isolates had shown them to contain identical plasmids. Nothing has been documented on the possibility of transmission of plasmids between C.pylori strains and it is not known if such a mechanism exists. Restriction enzyme analysis of the chromosomal DNA of the isolates with identical plasmids would reveal whether the organims were identical, or whether they were different organisms sharing identical plasmids. This information would be of epidemiological value as it might allow investigations into possible modes of transmission of C.pylori: or, if the plasmids appeared to have been transmitted to different C.pylori strains, this might aid the study of plasmid transmission between C.pylori isolates.

The method used for the extraction of chromosomal DNA from C.pylori was that described by Langenberg et al (1986) in their study on the identification of C.pylori isolates by restriction enzyme analysis. Isolates B1 and B2 were grown in 100ml of Brucella broth supplemented with 10% horse serum for 36 hours at 37^{o}C under microaerophillic conditions. The cultures were shaken at 100rpm to provide maximum aeration

of the broth. The cells were harvested by centrifugation in a J2-21 Beckman centrifuge (rotor JA20) at 6000rpm for 10 minutes at 4°C. The cells were washed once with phosphate buffered saline (pH 7,4) and twice with TE buffer (10mM Tris-HCl, pH8,5; 10mM EDTA) under similar centrifugation conditions as above. After suspending the cells in the latter buffer, they were treated with lysozyme at a concentration of 3mg/ml for 15 minutes at 37°C. Sodium dodecyl sulphate (1%) was added to lyse the cells and the tubes were left on ice for 20-30 minutes to ensure complete lysis. To remove RNA, RNase A (50µg/ml) was added and the tubes were incubated at 37°C for 60 minutes. This was followed by the addition of Pronase (0,75mg/ml) and overnight incubation at 50°C to denature proteins. The following day, 2,5ml of STE buffer (150mM NaCl; 10mM Tris-HCl, pH 8,5; 1mM EDTA) was added and the DNA was extracted with an equal volume of phenol/chloroform to remove proteins. Absolute ethanol (2,5 volumes) was added and this immeadiately resulted in the formation of a clot of DNA, which was spooled onto a glass rod and dissolved in 1ml of sterile distilled water. A second precipitation was done overnight in the presence of 2,5ml of absolute ethanol and at -20°C. The DNA was harvested by centrifugation at 10000rpm for 30 minutes and the pellet was washed with 5ml of absolute ethanol by centrifuging as described before. The

pellet was dissolved in 1ml of sterile distilled water and the DNA solution was quantitated on a spectrophotometer. For isolate B2, the yield of DNA was 70µg and the ratio of absorbance at 260nm to that at 280nm was 1,80, indicating that the DNA was relatively pure. Isolate B3 had an $absorbance_{260nm}$ to $absorbance_{280nm}$ of 1,89 indicating that this preparation was slightly less pure than that of isolate B2. Chromosomal DNA was digested with a variety of restriction enzymes to generate a restriction fragment pattern.

CHAPTER 7 ANALYSIS OF C.PYLORI CHROMOSOMAL DNA

Figure 8 shows the results of digestion of C.pylori chromosomal DNA with a variety of restriction enzymes. Lanes 8 and 9 represent undigested DNA of isolate B2 (3µg) and B3 (3µg) respectively, and as can be seen in lane 8, the concentration of plasmids in isolate B2 do not appear to be as low as was previously thought. This indicates that the method of plasmid extraction used up to now was inefficient in the recovery of intact plasmid DNA. Lanes 1 and 2, 3 and 4, and 5 and 6 represent BglI, HindIII and SmaI digests of chromosomal DNA from isolates B2 and B3, respectively. The most obvious feature of the photograph is that partial digestion has occurred in isolate B3 (lanes 2, 4 and 6). Close inspection of lanes 1 and 2 reveals almost identical restriction patterns, with the majority of differences being in the intensity of bands rather than the presence or absence of bands. Fragments which appeared in both isolates B2 and B3, but with different intensities are highlighted in lane 1. Lanes 3 and 4 appear to have identical HindIII restriction patterns, with different intensities of bands between isolates. The fragments appearing in both lanes 3 and 4, but with differing intensities are marked. The partial digestion of isolate B3 is very noticeable. It may be important to note that HindIII is sensitive to methylation of adenine and cytosine residues in its recognition site, while BglI and SmaI are sensitive only to

methylation of cytosine residues. If the methylation pattern favoured during growth of B3 resulted in the methylation of adenine and cytidine residues, then the activity of restriction enzyme HindIII would be inhibited more than that of the other two enzymes. Lanes 5 and 6 represent SmaI digests of isolates B2 and B3 respectively. This enzyme appears to have digested the chromosomal DNA into a large number of fragments and this has resulted in a smear rather than individual bands. All incubations were done at 37°C overnight, but when the digestion reactions were repeated and incubated for only 6 hours, most enzymes digested the DNA only partially.

The results of digestion of C.pylori chromosomal DNA are difficult to interpret as a large number of fragments are generated by the enzymes tested. Close inspection of the restriction enzyme patterns of the isolates tested, reveals that they have very similar patterns. Since the plasmids of these two isolate are very similar, it is likely that these two isolates are also very similar. At least two patients then, probably were infected with the same organism, and this may be of epidemiological value. The other three isolates showing the common plasmid profile appeared identical on the basis of size of colonies and rate of growth and it is possible that they are identical to the two studied here. No evidence exists in the available literature for a natural mechanism of plasmid transfer between different C.pylori

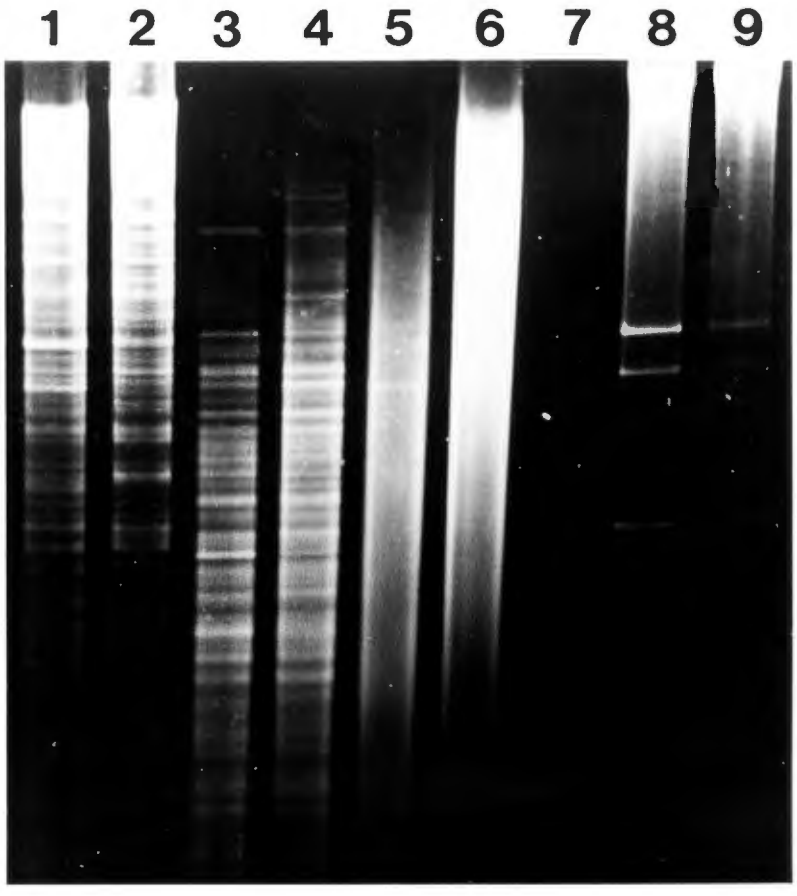


Figure 8: Restriction enzyme digestion of C.pylori chromosomal DNA. Lanes 1 and 2 show BglI digests of isolates B2 and B3 respectively. Similarly, lanes 3 and 4 show digestion with HindIII and lanes 5 and 6 show digestion with SmaI. Restriction patterns appear identical for isolates B2 and B3 except that partial digestion has occurred in isolate B3. Lanes 8 and 9 contain undigested DNA from these two isolates.

strains. In their work, Langenberg et al (1986) comment in the value of using restriction enzyme patterns of chromosomal DNA in epidemiological studies since they found different isolates had different restriction patterns. The finding in this work that methylation of DNA affects the restriction pattern, raises the question of whether the results of Langenberg et al (1986) were valid. It is possible that the differences they found were as a result of DNA methylation inhibiting digestion, rather than differences in restriction enzyme sites.Their results as well as the ones presented here are complicated, due to the large number of fragments; it is questionable whether this technique could be used routinely for differentiating strains of C.pylori.

CHAPTER 8 END LABELLING OF DNA

Low yields of DNA were obtained from small and large scale plasmid preparations. This made the construction of a restriction enzyme map difficult as DNA fragments were often not detectable after digestion and electrophoresis. An alternative method of detecting the DNA bands to staining them with ethidium bromide and illuminating with ultraviolet light was required. Radioactive end-labelling and detection by autoradiography was investigated as an alternative method. This method is more sensitive than detection by ethidium bromide staining and ultraviolet illumination. When radiolabelling of DNA fragments is used in conjunction with partial digestion, it can be a powerful tool for the construction of a restriction enzyme map.

8.1 PILOT EXPERIMENT TO ESTABLISH OPTIMAL CONDITIONS FOR END LABELLING

A pilot experiment was performed to establish the minimal amount of labelled DNA needed to give a good signal on autoradiography and to establish optimal conditions for the experiment. Varying concentrations of DNA (200ng, 100ng, 75ng and 50ng) were digested with HindIII. Each digest was then treated with 2 units of 'Klenow' enzyme, which has a 3'-5' exonuclease activity, at room temperature for 15 minutes. This was followed by the addition of 3µl of a

nucleotide solution (100μM dATP, 100μM dGTP and 100μM dTTP), 1μCi of [^{32}P]dCTP (specific activity- 3000 μCi)and by incubation at room temperature for 15-20 minutes. All the digests and end labelling reactions were done in duplicate and electrophoresed on the same agarose gel. Before electrophoresis, the reactions were terminated by heating the mixture to 65^{o}C for 5 minutes. After electrophoresis, the gel was cut in half, each half with a range of DNA concentrations. One half was dried under a vacuum at 70^{o}C for approximately 3 hours before being wrapped in plastic and placed at -70^{o}C for autoradiography for 3.5 hours. A Southern blot was prepared from the second gel after denaturation and neutralization of the DNA (see section 9.1). The membrane was wrapped in plastic and placed at -70^{o}C for autoradiography for 3.5 hours.

8.2 RESULTS OF PILOT EXPERIMENT

The first 15 minute incubation in the presence of Klenow enzyme resulted in the removal of nucleotides in a 3'-5' direction. The subsequent addition of nucleotides (including labelled dCTP) allowed synthesis of the complementary DNA strand by the 5'-3' polymerase activity of the enzyme. This resulted in the radioactive labelling of all fragments generated by restriction enzyme digests. The DNA was detected after electrophoresis by ethidium bromide staining

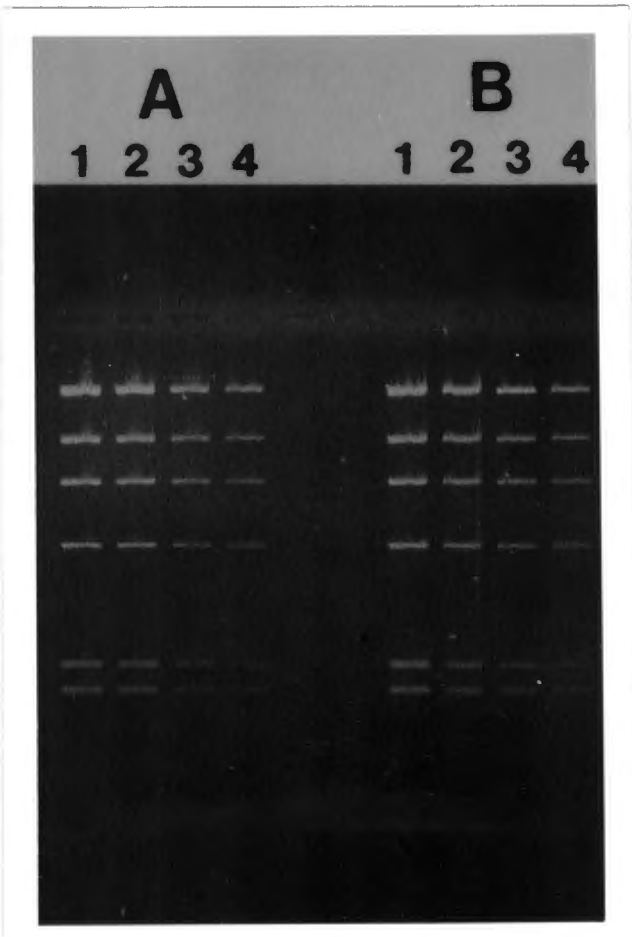


Figure 9
Agarose gel of lambda DNA digested with HindIII and end labelled. Lanes 1, 2, 3 and 4 contain 200, 100, 75 and 50ng of DNA respectively. A and B are identical and were separated to be used in a Southern blot and dried under a vacuum, respectively.

and ultraviolet illumination (see figure 9). The 0.5kb HindIII fragment of lambda DNA was barely detectable on the gel. A slight smearing of the bands indicated that some degradation of the DNA had occured during the labelling reaction, but fragments were still sufficiently intact to give good, sharp bands after autoradiography. A comparison of the two autoradiographs (see figures 10a and 10b) showed both methods to be much more sensitive for DNA detection than ethidium bromide staining. The 0,5kb HindIII fragment was clearly visible in both, although the dried gel had cracked on release of the vacuum, resulting in a slightly distorted image on the X-ray film. From the autoradiograph of the DNA transferred to the nylon membrane, it was apparent that diffusion of the bands had occured. In both autoradiographs, the background was low. A slight background signal visible on the autoradiograph made from the dried gel was most likely due to the type of plastic used to wrap the gel for autoradiography, rather than unincorporated labelled nucleotides present in the gel. These labelled nucleotides should have been separated from the DNA during electrophoresis and probably migrated right off the gel due to their small size. It is not clear why small fragments gave a weaker signal on autoradiography than larger ones. The nature of the labelling reaction is such that all fragments should be labelled to the same intensity as all ends generated by restriction enzyme digestion were

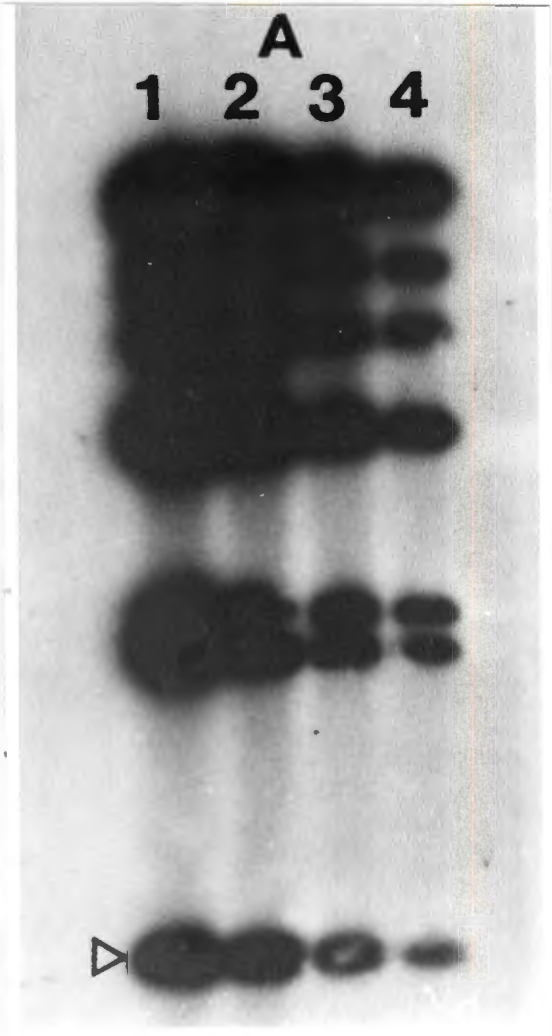


Figure 10a: Autoradiograph of gel A in Figure 9, which was blotted onto nitrocellulose The arrow denotes the 0,5kb fragment barely visible on the gel.

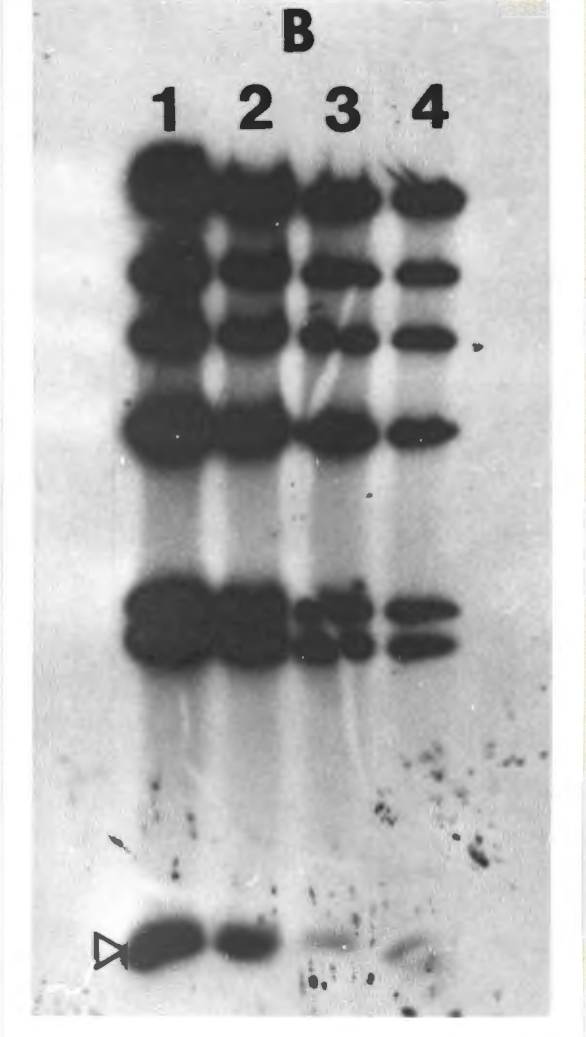


Figure 10b: Autoradiograph of gel B from figure 9, which was dried under a vacuum. The arrow denotes the same fragment as in Figure 10.

subjected to the DNA synthesis reaction for the same length of time.These results showed that end labelling is a highly sensitive DNA detection system, detecting 50ng of digested DNA with ease. The method is quick, the labelling reaction can be performed immediately after the restriction enzyme digestion without changing the buffer, and the gel is dried, and exposed to X-ray film for autoradiography.

8.3 END LABELLING OF C.PYLORI DNA

To construct a restriction enzyme map of the common plasmids of C.pylori isolates, the plasmids were digested with the enzymes EcoRI and HindIII. Single and double digests were done and the labelling reaction was performed at various stages of the digests. When the labelling reaction was done in between two digests, it was stopped by heating to 68^{o}C for 10 minutes before addition of the second restriction enzyme. The activity of the second restriction enzyme was stopped by the addition of stop buffer (see section 5.3).

In a second experiment, the covalently closed circular plasmid was gel purified and digested with EcoRI and HindIII individually and also with the two enzymes simultaneously. After digestion, each of these restriction enzyme fragments were end labelled as previously described. In this experiment though, the incubation time in the presence of

Klenow enzyme before the addition of the nucleotides, was increased to approximately 25-30 minutes.

All lambda markers were digested with HindIII and end labelled as above. In the second experiment however, the incubation time of lambda DNA with Klenow enzyme and no nucleotides was only 10 minutes. Digested C.pylori and lambda DNA were electrophoresed in agarose gels, stained with ethidium bromide and photographed over ultraviolet light. This was followed by drying the gels under a vacuum at 75-80^{o}C for 1-2 hours and by autoradiography.

8.4 RESTRICTION MAPS GENERATED BY END LABELLED C.PYLORI DNA

i) End labelling between double digests

Figures 11 and 12 show a photograph of the ethidium bromide stained gel and an autoradiograph of the dried gel respectively. It is clear from the photograph of the gel that both lambda DNA markers were degraded and thus no estimation of the size of the C.pylori fragments was possible. When the bands are compared with similar digests shown in figure 5, the number and positions of the fragments suggests that partial digestion had occured. The characteristic doublet (1.2kb and 1,3kb) of previous HindIII digests is marked on the photograph and it is probable that the two largest bands visible on the photograph and on the

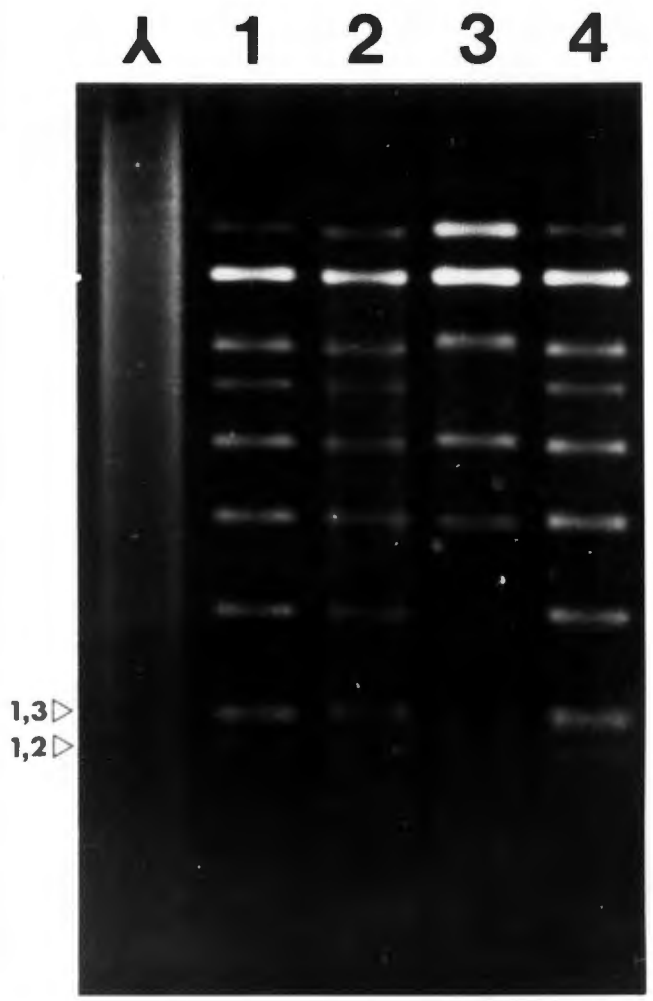


Figure 11: Agarose gel of digested, end-labelled, C.pylori DNA. Lane 1-EcoRI, HindIII digestion followed by end-labelling; Lane 2- EcoRI digestion, end-labelling, then HindIII digestion; Lane 3- HindIII digestion, then end-labelling; Lane 4- HindIII digestion, end-labelling, then EcoRI digestion;

lanes 1, 2 and 4 all show identical fragments, suggesting that end-labelling continued throughout the second digest.

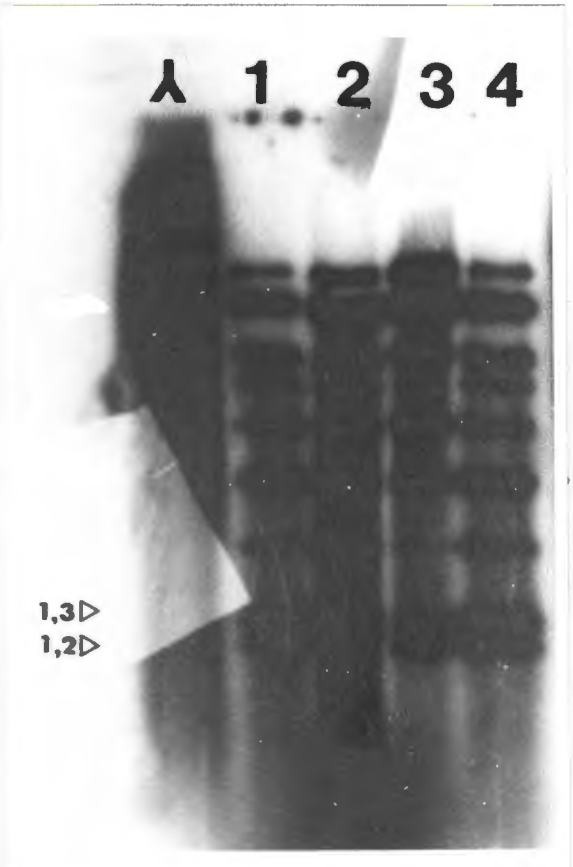


Figure 12: Autoradiograph of gel shown in figure 11. Lanes 1,2,3 and 4 are as described above. The smear seen in all lanes is as a result of shearing of chromosomal DNA.

autoradiograph represent undigested DNA. The two fragments present in lane 4 which are absent in lane 3 appear to be generated by EcoRI digestion of the largest and/or second largest bands seen in the HindIII digest.

ii) End labelling after digestion

Very little DNA was visible on this gel. Nevertheless, the gel was dried and left at -70°C for 30 minutes for autoradiography (see Figure 13). The DNA appears to be severely sheared and this makes it difficult to identify fragments generated by digestion. The DNA that was digested here was gel purified covalently closed circular DNA. After digestion with EcoRI only the two slower migrating bands of the 3 from the common plasmid profile were visible (lane C). This could either have been due to damage to the DNA during gel purification, resulting in nicked and linear DNA, or there could be one EcoRI site on this plasmid which results in a linear fragment after digestion. The nicked plasmid band could be the result of incomplete digestion. Digestion of this plasmid with HindIII resulted in an identical profile to the previous end labelled HindII digest (lane B). The characteristic doublet (1,3kb and 1,2 kb) is visible as well as the other fragments resulting from a partial HindIII digest. Once again, the two largest fragments of the HindIII digest appear to have been digested by EcoRI when the DNA is digested simultaneously with these two enzymes (lane A). An important feature of the autoradiograph is that the 125bp

fragments are visible in the lambda HindIII digests. These fragments are not visible in any of the other autoradiographs. It is important to note that the time of incubation for the exonuclease activity of the Klenow enzyme was only 5-10 minutes for lambda DNA in this experiment as opposed to 15-20 minutes in other experiments. It is possible that in a 15-20 minute incubation the exonuclease digests small fragments entirely so that a 125bp fragment is visible only with a shorter incubation time. This would also explain why a 500bp fragment was visible in figure 18 where fragments were detected by hybridization with a labelled probe, but not on any of the end labelled samples.

Although end labelling is a powerful tool for detecting low concentrations of DNA, the problem of obtaining complete digestion with EcoRI and HindIII and the failure of the step intended to stop the labelling reaction inbetween digests made the construction of an accurate restriction map difficult. (see page 78A for a provisional restriction map).

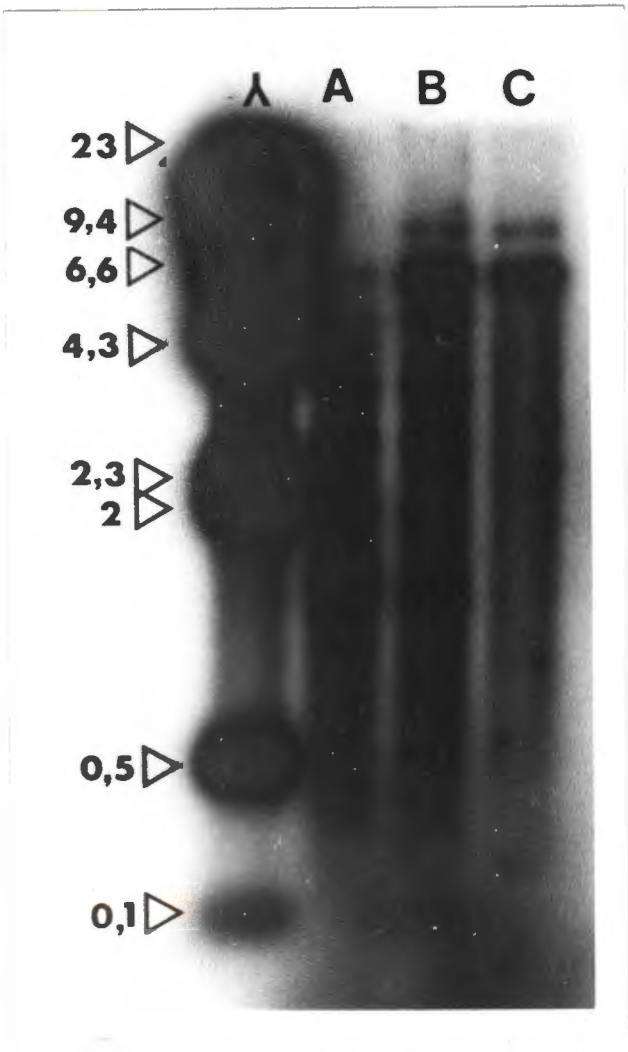


Figure 13: Autoradiograph of digested, end-labelled C.pylori DNA. Individual fragments are difficult to differentiate from sheared DNA in the background. Lane A shows a double digest with HindIII and EcoRI; Lane B shows the results of a HindIII digest; and lane C, an EcoRI digest. End-labelling was done after digestion. Notice the 125 bp fragment detected by HindIII digestion of lambda DNA. Sizes are given in kilobase pairs.

CHAPTER 9 HYBRIDIZATION

Hybridization involves the formation of a double helix of DNA from two complementary strands; it is based on the principle that two pieces of DNA with similar base sequences will, under the correct conditions, form hydrogen bonds between their bases and so hybridize to each other. According to Britten and Davidson (1985), the formation of the duplex is dependant on complementarity between the sequences involved. The stability of such a duplex depends on the extent of complementarity ie. the greater the complementarity, the more stable the duplex. Hybridization techniques can be applied to a wide range of biological problems, but in this work, it was used to establish how closely related various fragments of DNA were. Whole plasmids, as well as restriction enzyme fragments of DNA, were separated by agarose gel electrophoresis. The DNA was denatured and transferred to nylon membranes by a modified method of Southern (1975), which is described below. The membranes were probed with radiolabelled DNA which bound to similar sequences on the membrane. Those fragments which contained similar base sequences to those of the probe DNA could be detected by autoradiography.

9.1 TRANSFER OF DNA TO NYLON MEMBRANES

Prior to transfer, DNA fragments (total plasmid DNA or restriction enzyme fragments) were separated by agarose gel electrophoresis. If the DNA fragments were larger than 10kb, the gel was immersed in 0,25M HCl for 15 minutes at room temperature after electrophoresis to hydrolyse the DNA and generate smaller fragments. This ensures more efficient transfer of the DNA onto the membrane. The DNA was denatured in 500ml of denaturing solution (1,5M NaCl and 0,5M NaOH) for 30 minutes and this was replaced with fresh solution for a further 30 minutes. This was followed by 2 similar 30 minute incubations in neutralizing solution (1,5M NaCl; 0,5M Tris-HCl, pH 7,2; 0,001M EDTA) at room temperature. To facilitate the diffusion of these solutions into the gel, the gel was placed on a rocking apparatus during the denaturation and neutralization steps.

For the transfer of DNA to a Hybond-N membrane, 3 pieces of 3MM blotting paper which were larger than the gel, were soaked in 20 x SSC buffer (1 x SSC is 0,15M NaCl; 0,15M sodium citrate, pH 7,0) and laid on a piece of plastic on the benchtop. The gel was placed well-side down on the 3MM paper and a piece of Hybond-N, cut to the same size as the gel, was laid onto the gel carefully, ensuring that no air bubbles were trapped between the two. Next, a sheet of 3MM paper cut to the same size as the gel was placed on top of

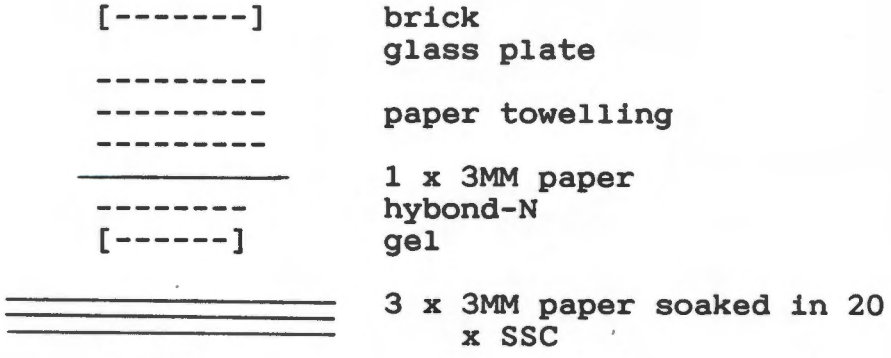


Figure 14: Diagram of DNA transfer apparatus.

the nylon membrane followed by a thick wad of absorbent towel. The assembly was flattened and weighed down with a glass plate and a brick, on top of the absorbent towel (see Figure 14). The transfer was allowed to proceed overnight and the towels were replaced at least once as soon as they were wet.

After the transfer, the membrane was wrapped in plastic and illuminated, DNA-side down, on an ultraviolet transilluminator for 10-15 minutes. This step cross-linked the DNA irreversibly to the membrane.

9.2 PREPARATION OF THE PROBE

DNA to be used as a probe was gel purified by the method described in Appendix 2. Two alternative methods were used for radiolabelling of the DNA - the first described in the manual supplied with the Nick Translation Kit (Amersham No.N5000) and the second described in the manual supplied with the Multiprime DNA Labelling Systems (Amersham No. RPN1601).

For nick translation, 0,5-1µg of DNA was labelled. The reaction contained the DNA, suspended in 65µl of distilled water, 20µl of buffer containing 100mM dATP, 100mM dGTP and 100mM dTTP, and 5µl of [^{32}P]dCTP (50µCi). To this mixture,

10µl of enzyme solution, which contained DNA polymerase I (5 units) and DNase I (100pg), was added and thoroughly mixed with the contents in the tube. The tube was centrifuged in a microfuge for a few seconds to collect the contents at the bottom and then incubated at $15^{o}C$ for 1.5 hours. This reaction should yield a probe with a specific activity of $1x10^{8}$ dpm/µg of DNA.

When using the Multiprime DNA Labelling System, the plasmid DNA to be used as a probe was first linearised by digestion with EcoRI. A maximum of 25ng of DNA was used in the labelling reaction and this was dissolved in 5µl of sterile distilled water in an Eppendorf tube. The DNA was denatured by placing the tube in a boiling water bath for 5-10 minutes and then chilling it on ice to prevent renaturation. All reagents for the reaction were thawed and mixed in an ice bath. To 5µl of the linearised, denatured DNA, the following were added: 10µl of buffer containing unlabelled dATP, dGTP and dTTP, 5µl of primer solution consisting of random hexanucleotides, 23µl of sterile distilled water, 5µl of [^{32}P]dCTP (50µCi) and 2µl (2 units) of enzyme. The enzyme used was the 'Klenow' fragment of DNA polymerase I which lacks the 5'-3' exonuclease activity associated with DNA polymerase I. This ensured that incorporated labelled nucleotides were not subsequently removed. The solutions were mixed by pipetting up and down and the tube was centrifuged for a few seconds to collect the contents at the

bottom. The reaction was allowed to proceed at room temperature for 3 hours. This method should yield a probe with a specific activity of $1,8x10^9$ dpm/µg of DNA.

9.3 REMOVAL OF UNINCORPORATED NUCLEOTIDES FROM THE PROBE

Unincorporated labelled nucleotides have to be separated from the probe DNA because they may bind to the membrane. This would result in a high background level of radioactivity. Two methods were used for this:

9.3.1 SPUN COLUMN FILTRATION

A Tuberculin (1ml) syringe was stoppered with siliconized glass wool. The syringe was filled with Sephadex G50 (in distilled water) and centrifuged at 1500 rpm for 4 minutes. More Sephadex G50 was added and the centrifugation repeated until the Sephadex beads reached the 0,8ml mark. During centrifugation, the tip of the syringe was placed in an Eppendorf tube to collect the water. Nick translation buffer (50mM Tris-HCl, pH 7,5; 150mM NaCl; 10mM EDTA; 0,1% SDS) was used to wash the column by centrifugation as before. After incubation of the labelling reaction, the sample was mixed with 4µl of stop buffer and loaded onto the column, followed by 200µl of nick translation buffer. The column was centrifuged as before and the probe was collected in an Eppendorf tube.

9.2.1 SETTLED COLUMN FILTRATION

The narrow glass end was broken off the tip of a Pasteur pipette and a sterile glass bead was placed in the narrow end of the pipette to block that end partially. The tip of the pipette was placed in an Eppendorf tube and the pipette was filled with Sephadex G50 (in water). Once the beads had settled, 100µl of nick translation buffer was loaded onto the column and a 100 µl aliquot was collected in the Eppendorf tube. After incubation of the labelling reaction, 100µl of the reaction mixture (the Multiprime reaction was made up to this volume with sterile distilled water) was mixed with the appropriate volume of stop buffer and loaded onto the column. An aliquot of 100µl was collected again in a fresh Eppendorf tube. Aliquots of 100µl of nick translation buffer were washed through the column and collected in sequential Eppendorf tubes. Once 15 aliqouts had been collected, a Geiger counter was used to determine which tubes contained the radioactively labelled probe. The contents of the 4 tubes registering the highest level of radioactivity were pooled and used as the probe.

The radioactive probes were denatured by heating to 95^{o}C for 5-10 minutes before hybidization.

9.4 HYBRIDIZATION

This was done as described by Johnson et al (1984). Prior to hybridization with the radioactively labelled probe, a prehybridization step was performed. This involved washing the membrane with 20ml of a prehybridization solution (6ml of 20xSSC, 14ml of distilled water and 0,05g of non-fat milk powder) in a sealed plastic bag at 42°C for at least 2 hours. This step reduces non-specific binding of the probe to the membrane. After prehybridization, the bag was cut open and all the liquid was removed. The denatured probe, together with 10ml of hybridization solution (3ml of 20xSSC, 5ml of formamide, 2ml of distilled water and 0,025g of non-fat milk powder), were pipetted into the bag which was re-sealed. The formamide destabilises the DNA double helix and therefore prevents renaturation of the probe. This allows hybridization to be carried out at the relatively low temperature of 42°C overnight. After hybridization, the hybridization solution was removed and the membrane was washed with increasing stringency to remove probe DNA that had bound non-specifically to the membrane and to DNA with less than 90% homology to the probe. Wash solution 1 (2xSSC, 0,1% SDS and 0,25% non-fat milk powder) was used for two 30 minute washes, each with 500ml, at room temperature. This was followed by two similar washes with wash solution 2 which has a lower salt concentration (0,1xSSC and 0,1% SDS) at 55°C, thus increasing the stringency of the wash.

After the final wash, the membrane was placed in a fresh plastic bag, which was sealed, and then placed at -70^{o}C for autoradiography (Film- Cronex, type4, double side emulsion).

9.5 RESULTS AND DISCUSSION OF HYBRIDIZATION EXPERIMENTS

A range of C.pylori plasmids were probed with a probe consisting of gel purified, covalently closed circular plasmid from one of the isolates with a common plasmid profile. All C.pylori plasmid bands gave signals on the X-ray film except one isolate which contained plasmids of greater than 23kb. The absence of a signal from this isolate may be due to poor transfer of the DNA to the membrane as a result of the very large size of plasmids in this isolate. Various interpretations of these results are possible. Either all the plasmids of the isolates examined contain related sequences which may or may not carry functional genes. The other explanation for all of the C.pylori plasmids giving a signal on autoradiography is that non-specific binding of the probe to the DNA on the membrane may have occurred and insufficiently stringent washes failed to remove this probe. In order to determine which of these theories applied, another experiment was be done where the membrane that was probed contained unrelated DNA to serve as a negative control for hybridization. For this experiment the gel that was blotted contained laboratory constructed

plasmids used as size markers for negative controls for hybridization. However, after autoradiography it was revealed that all of these markers except two also gave a positive signal when probed with covalently closed circular DNA from C.pylori isolate B2. An investigation of the origin of plasmid markers revealed that all markers which hybridized to C.pylori DNA originated from the plasmid pUC19, a pBR322 derivative. This implied a sequence similarity between the C.pylori plasmids and pUC. To determine which region of pUC DNA was hybridizing to C.pylori DNA, the pUC DNA was digested with RsaI, RsaI and BglI, and RsaI and PvuII. The digested DNA was electrophoresed, blotted onto a nylon membrane and probed with nick translated C.pylori plasmid DNA from isolate B2. Figure 15 shows the electrophoretic migration pattern of digested pUC DNA. Not all of the expected fragments are visible on the gel and this could either be due to the size of the missing fragments (very small fragments may have so little bound ethidium bromide that they are not detectable) or the small fragments may have migrated off the gel. Figure 16 shows the result of the hybridization after 2 weeks of autoradiography. A very weak signal is visible from the three pUC fragments containing the origin of replication (marked by arrows in Figures 15 and 16), suggesting homology between the C.pylori plasmid and the origin of replication of pUC. When the experiment was reversed, ie. pUC DNA was

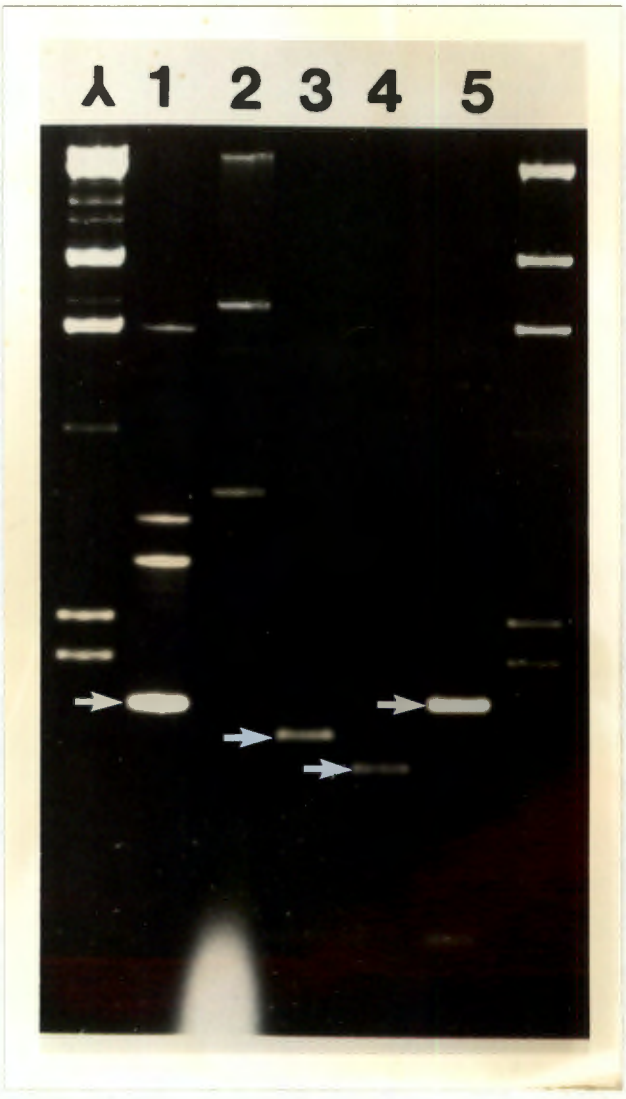


Figure 15: pUC 19 DNA digested RsaI and PvuII (lane 3), RsaI and BglI (lane 4) and RsaI (lane5). Lane 1- undigested pUC; Lane 2- undigested C.pylori DNA. Fragments that gave signals on autoradiography are marked by arrows.

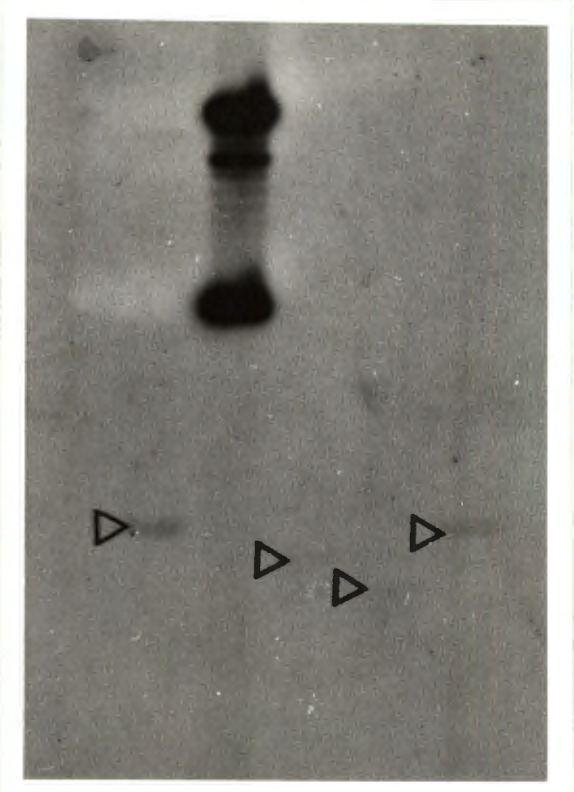


Figure 16: Autoradiograph of the gel shown in figure 15. Arrows denote those fragments of pUC that gave signals on autoradiography.

used to probe membrane bound C.pylori DNA, no signal was obtained after 2 days of exposure. The results obtained in this experiment are dependant on the time of autoradiography and the positive signal obtained is probably more as a result of prolonged exposure than a true indication of homology between pUC DNA and C.pylori DNA. If the results are significant, this could have important implications in the cloning of C.pylori plasmids into pUC vectors, because if they belong to the same incompatiblity group, this could affect their stability in the same host cell.

Restriction enzyme fragments which were difficult to detect by ethidium bromide staining of agarose gels, were more easily detected by hybridization techniques or end-labelling. The gel shown in figure 5 was blotted onto nitrocellulose and probed with plasmid DNA isolated from C.pylori isolate B2. When compared with the autoradiograph shown in Figure 17 it can be seen that a number of very faint bands on the gel are shown clearly on the autoradiograph. The autoradiograph also confirms the presence of a fragment of approximately 500bp in the double digests using EcoRI and HindIII as well as the digests using HindIII alone.

The technique of hybridization is more sensitive in the detection of DNA than ethidium bromide staining and ultraviolet illumination and detects only homologous DNA sequences.

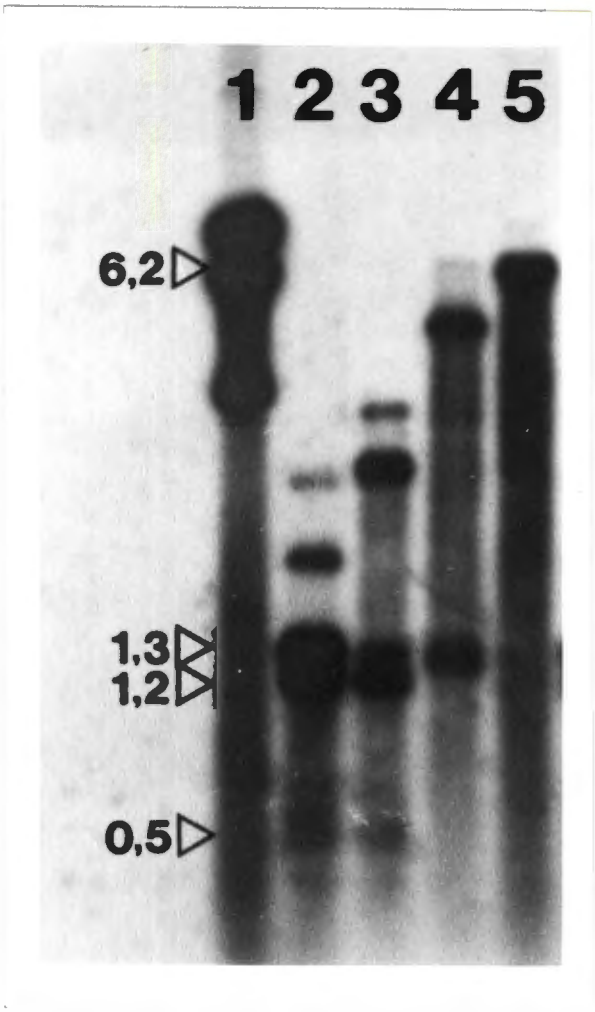


Figure 17: Autoradiograph of the gel shown in figure 5, using C.pylori DNA as a probe. Note the 0,5kb fragment present in the HindIII digest which is detectable by hybridization, but not by ethidium bromide staining of the gel.

CHAPTER 10 CLONING

Throughout this work difficulties were experienced in purifying large amounts of C.pylori plasmid DNA. This hampered much of the work, especially the construction of a restriction enzyme map and the investigation of relationships between plasmids.

Chapter 3 describes the specific requirements for the growth of C.pylori. Many of the plasmids of C.pylori have a low copy number ie. only a few copies of the plasmids exist in each cell. If these plasmids could be cloned into a vector with a high copy number and the recombinant plasmids used to transform an aerobic host cell which grows quickly at 37^{o}C, much higher concentrations of C.pylori DNA could be obtained.

Vectors are engineered plasmids which contain a number of single restriction enzyme sites which can be used to clone foreign DNA into them. Once these vectors contain inserts of foreign DNA, they can be used to transform host cells in a system which allows the selection of cells containing a recombinant plasmid.

10.1 VECTORS AND HOST CELLS

10.1.1 VECTORS USED FOR CLONING EXPERIMENTS

The vectors used for the construction of recombinant plasmids were pUC 18 and pUC 19 (see Appendix 4 for restriction enzyme maps). These vectors have a high copy number ie. once inside the host cell they replicate rapidly until a large number of recombinant plasmids exist in the cell. They are derived from plasmid pBR322 and both contain a multiple cloning site which has single sites for a number of restriction enzymes. The multiple cloning site lies within a region encoding part of the B-galactosidase gene and the only difference between the two vectors is the orientation of the multiple cloning site. If insert DNA is cloned into the multiple cloning site, the B-galactosidase gene is interrupted and no functional enzyme is produced. Transformed JM109 cells, when grown in the presence of X-gal (5-bromo-4-chloro-3-indolyl-B-galactoside), IPTG (isopropyl-B-D-thiogalactopyranoside) and ampicillin, will be blue, as a result of the hyrolysis of X-gal by the B-galactosidase enzyme to bromochloroindole, a blue dye, if the vector does not contain an insert. A recombinant vector, however, will not produce a functional B-galactosidase enzyme and transformed host cells will therefore be white. The pUC vectors also contain a B-lactamase gene, encoding ampicillin resistance. Transformed host cells are grown on luria agar

containing ampicillin, X-gal and IPTG and this allows the selection of cells containing recombinant plasmids.

10.1.2 HOST CELLS USED FOR TRANSFORMATION EXPERIMENTS

The host cells used are derivatives of E.coli K12, designated JM109. These cells have a genotype recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, λ^-, Δ(lac-proAB), [F', traD36, proAB, $lacI^qZ\Delta M15$] and restriction$^-$ and modification$^+$. Essentially, they contain part of the B-galactosidase gene which is complemented by the incomplete B-galactosidase gene encoded by pUC on an F' plasmid. The chromosomal deletion of the pro gene ensures the selection of plasmid containing cells on glucose medium and the traD36 mutation conforms with the NIH (US) recommendations for laboratory strains (Yanisch-Perron 1985).

10.2 PREPARATION OF VECTOR AND INSERT DNA

The multiple cloning site of pUC vectors contain single restriction enzyme sites for EcoRI and HindIII (see Appendix 4). Since these enzymes were shown to digest the common plasmids of C.pylori, the vectors were digested with either of these. The cohesive ends that were generated would normally be able to religate under ligation conditions, reducing the amount of available vector and thus diminishing the number of recombinants. To prevent this, the digested vector was treated with an enzyme which removes the 5'

phosphate groups of the cohesive ends, thus preventing recircularization of the vector.

After digestion of 1µg of pUC18 with the appropriate enzyme, the enzyme activity was terminated by a phenol/chloroform extraction and the DNA was precipitated with ethanol. The DNA pellet was resuspended in 17µl of sterile distilled water and 2µl of 10x CIP buffer (100mM Tris-HCl, pH 9,2; 1mM EDTA) was added. The enzyme used was calf intestinal phosphatase (CIP) (1 unit) and the reaction was allowed to proceed for 15 minutes at 37°C. A phenol/chloroform extraction terminated the reaction and this was followed by precipitation of the DNA by ethanol. For most cloning experiments, the alkaline phosphatase step was omitted as the enzyme was shown to have some exonuclease activity and thus damage the cohesive ends of the vector, preventing ligation with the insert.

The insert DNA was digested with the same enzyme as had been used for the vector. A phenol/chloroform extraction was performed to remove the enzyme, the DNA was precipitated with ethanol and resuspended in 10µl of sterile distilled water.

10.3 LIGATION

Before ligation, an aliquot of the vector and insert solutions were electrophoresed to estimate their DNA

concentrations. This was important as the ratio of vector to insert DNA affects the efficiency of ligation. For the ligation experiments, a vector to insert ratio of 1:3 was used. Generally, the ligations contained approximately 35ng of vector DNA and 105ng of insert DNA. To this mixture, the appropriate amount of 10x ligation buffer (660mM Tris-HCl, pH7,2; 10mM EDTA; 100mM $MgCl_2$; 100mM dithiothreitol and 1MM ATP) and 1 unit of T_4 DNA ligase were added. The ligation mixture was usually in a total volume of 10µl and it was incubated overnight at 15°C. In one experiment, the ligation reaction proceeded for only 4 hours, but this appeared to result in incomplete ligation which decreased the efficiency of transformation so all further ligations were incubated overnight.

Ligation controls were included in these experiments. To check the efficiency of the ligation reaction, linearised pUC18 was incubated in the presence and absence of T_4 DNA ligase.

10.4 TRANSFORMATION

After overnight ligation, the ligation mixture was used to transform competent JM109 host cells. The technique used for the preparation of competent JM109 cells is that described by Hanahan (1983). The cells were prepared in batches and discarded if their competence was less than $1x10^6$. For transformation, 200µl of frozen competent cells were thawed

in Eppendorf tubes on ice for 20 minutes. Half of the ligation mixture was added to the cells and the tubes were incubated on ice for a further 30 minutes. The cells were heat shocked at 42°C for 2 minutes. This was followed by the addition of 1ml of prewarmed luria broth and incubation for 1 hour at 37°C to allow expression of the gene encoding ampicillin resistance carried by the vector which is used for the selection of recombinant colonies. The cells were then plated onto selective medium containing 250µl of X-gal (20mg/ml in dimethylformamide), 50µl of IPTG (23,8mg/ml in distilled water) and 100µl of ampicillin (50mg/ml) per 100ml of luria agar. The plates were incubated overnight at 37°c. Recombinant colonies were identified by their white colour, as opposed to the blue of colonies which did not contain recombinant plasmids.

Transformation controls were included in these experiments. Transformation efficiencies in the range of $5x10^6$ to $1x10^8$ were obtained. Undigested pUC was used to check the efficiency of transformation.

10.5 RESULTS AND DISCUSSION OF CLONING EXPERIMENTS

In the first cloning experiment, the gel purified, 6,2kb EcoRI plasmid DNA from isolate B2 was used as insert DNA. The vector used was pUC18 and it was treated with CIP before ligation. Despite this , an excess of blue colonies appeared on the selection plates. Thirty six white colonies were

plated onto luria agar containing ampicillin (to ensure retention of the plasmid) for storage. They were also grown up overnight in luria broth and ampicillin for investigation by the rapid, small scale technique of plasmid extraction. None of these colonies were shown to contain insert DNA after digestion of the plasmids and electrophoresis. These false recombinants (white colonies with no insert DNA) were most likely a result of damage to the B-galactosidase gene by exonuclease activity of the CIP enzyme, and subsequent religation of the vector.

In the second cloning experiment, the same plasmid as before was digested with either EcoRI or HindIII in an attempt to clone whole plasmid or fragments of the plasmid into pUC19. After transformation, most colonies were a faint blue colour, but after 6-7 hours of refrigeration, the intensity of the blue colour increased, allowing identification of 22 white colonies. These colonies were replica plated onto selective medium containing ampicillin, X-gal and IPTG and incubated overnight to identify true white colonies. Those that remained white (10) were examined for the presence of insert DNA. Plasmid DNA recovered after small scale plasmid preparation was digested with the enzyme used to restrict insert and vector DNA. This would linearise the vector, and separate it from the insert DNA. The digested DNA was electrophoresed to identify insert DNA.

Five recombinants contained foreign DNA, but none of the fragments were of the appropriate size that should have been generated by the initial digestion of the C.pylori DNA. This aroused suspicion as to whether these recombinants contained C.pylori DNA or whether the inserts represented contaminants. To test this, the recombinant plasmids were used to probe a Southern blot containing C.pylori DNA. This revealed that these inserts were contaminating DNA as no signal was obtained on the autoradiograph. It is probable that these DNA fragments were C.pylori chromosomal DNA present in the insert DNA preparations. Most of the DNA preparations contained contaminating chromosmal DNA as can be seen by the smear of DNA on almost every agarose gel.

Although treatment of the vector with CIP enzyme is supposed to prevent recircularization so that no blue colonies are obtained, a large number of these colonies occured. This could be the result of either an inactive CIP enzyme; or limited exonuclease activity of this enzyme preparation may result in recircularization of the vector with minimal damage to the B-galactosidase gene, resulting in the production of an active B-galactosidase gene.

CHAPTER 11 CONCLUSION

The finding that approximately 50% of the C.pylori isolates examined in this study contained plasmid DNA corresponds to the findings of Tjia et al (1987) and Majewski and Goodwin (1988). The majority of the isolates studied here had unique plasmid profiles, which confirms the findings of Tjia et al (1987). Differences in plasmid profiles have been found to correspond to serologically differentiated strains of C.jejuni (Bradbury et al 1983), but since no seroptyping system exists for C.pylori and only 50% of isolates contain plasmid DNA, another method of strain differentiation has to be devised for C.pylori. In their work, Majewski and Goodwin (1988) studied restriction patterns of chromosomal DNA from C.pylori isolates. They found a great variety of restriction patterns amongst the isolates. Langenberg et al (1986) also found that isolates from 16 patients gave different DNA digestion patterns. On the basis of these results they concluded that this technique is a sensitive method for identifying C.pylori isolates and suggest that it may be valuable in epidemiological studies. However, as is pointed out by Majewski and Goodwin (1988), the great variety of restriction patterns may mean that isolates are too diverse at the molecular level for this technique to be useful as a typing system. Also, the finding in this work that methylation is responsible for minor differences in restriction patterns suggests that this must be taken into

account when differentiating strains on the basis of restriction enzyme patterns. In this work, isolates with identical plasmid profiles were investigated further to determine whether they were the same strain. Chromosomal DNA restriction patterns with HindIII, BglI and SmaI were very similar in the two isolates examined, indicating that they may be of the same strain. It is likely that the third isolate with the common plasmid profile is identical to these two isolates, as it showed no differences in morphology or biochemistry.

Two of the three isolates with a similar plasmid profile came from adult patients, one of whom had a gastric ulcer, while no records are available on the second patient. The third isolate came from a paediatric patient with chronic gastritis. C.pylori were cultured from 13 patients whose gastric mucosa appeared normal on endoscopy. Out of 7 of these isolates that were examined for plasmid DNA, 4 were positive. Examination of the clinical data from all patients did not reveal any correlation between the type of disease caused (chronic gastritis, gastric ulcer or duodenal ulcer), and the presence or absence of plasmids. The three isolates with a common plasmid profile were morphologically and biochemically identical and no unusual features were noted which could have been attributed to the presence of a common plasmid.

The difficulties experienced in trying to purify high concentrations of plasmid DNA hampered the molecular studies. There is much evidence to suggest that the three bands seen in the common isolates represent alternative conformations of the same plasmid. Tjia et al (1987) also found in their study that some plasmid bands represented conformers of the same plasmid, suggesting that the plasmids are labile.

The labile nature of these plasmids resulted in difficulties in isolating covalently closed circular plasmids. The procedures involved in the gel purification of covalently closed DNA invariably resulted in conversion of the plasmid to open circular or linear DNA. The size of the covalently closed circular plasmid studied was estimated at 6,2kb on the basis of fragments generated by restriction with HindIII and EcoRI enzymes. A wide range of enzymes was used to digest the DNA, but the plasmids appeared refractory to digestion by all the enzymes tested except EcoRI, HindIII and Sau3A. On the basis of digestion of the C.pylori plasmid DNA common to the isolates by the isoschizomers Sau3A and MboI, the DNA appears to be methylated. This could explain the lack of digestion of the DNA by so many enzymes. Labigne-Roussel et al (1987) and Miller et al (1988) both give evidence for the presence of DNA modification and restriction systems in C.jejuni.

The restriction enzyme pattern obtained by digestion with Sau3A was unusual in that it contained 2 fragments of 2,2kb and 2,4kb each. Theoretically, one would have expected a large number of fragments of approximately 256bp in size after digestion with this enzyme (assuming that the DNA contained 50% guanosine and cytidine). The presence of these two larger fragments could indicate that the plasmids contain repetitive elements; this could also explain why so few enzymes were able to digest the DNA if the sequence of bases comprising the repetitive element did not contain any restriction sites. It would be interesting to know whether these two fragments show any homology to chromosomal sequences, as repetitive elements are usually located on the chromosome, and Campylobacter plasmid DNA has been reported to be related to chromosomal DNA (Tenover et al 1985).

In an attempt to visualize all the bands generated by digestion of the plasmids with restriction enzymes, fragments were end-labelled before electrophoresis. The electrophoresis gel was dried and an autoradiograph was made. This technique was shown to be more sensitive than ethidium bromide staining for the detection of DNA. The relationship between C.pylori plasmids was studied by DNA hybridization. All the plasmids appeared to be related to one another when hybridization and washing were done under conditions of high stringency (more than 90% homology was

required between probe and membrane bound DNA). Although the C.pylori probe DNA appeared to have bound to pUC19 after prolonged exposure of the membrane to the X-ray film (2 weeks), when pUC DNA was used to probe a membrane containing C.pylori DNA, no signal was obtained. In the latter experiment the exposure time was only 2 days; this raises the question as to whether a limit should be set on exposure time for this type of experiment, as results of homologous probing may be dependent on this factor.

Numerous attempts were made to clone C.pylori DNA into pUC vectors. When restriction enzyme results suggested that EcoRI cut the common plasmid once, the whole linear plasmid was used in an attempt to create a recombinant plasmid in E.coli host cells. None of the cloning experiments were successful.

The difficulties experienced in trying to clone C.pylori DNA are echoed by a report from Labigne-Roussel et al (1987) who cite the difficulties encountered in attempts to maintain and express Campylobacter genes in E.coli hosts as a major barrier to the genetic analysis of Campylobacter species. In an attempt to overcome this problem, they set about constructing a shuttle cloning vector. This chimeric plasmid was derived from pBR322 and contained Campylobacter replication functions, a kanamycin resistance gene and the origin of transfer from a broad host range plasmid. The vector can be mobilized from E.coli to C.jejuni, C.coli and

C.fetus by complementation with the transfer functions of an IncP plasmid at a frequency of 10^4 transconjugants per donor. The authors suggest three barriers which may account for the failure of gene exchange between these species. The first is that replication or expression of genes is inhibited, as the authors showed that conjugative transfer of the chimeric plasmid containing oriT sequences from an IncP plasmid did occur between E.coli and Campylobacter cells. Secondly, they found that Campylobacter cells harbouring the chimeric plasmid did not express the TEM beta-lactamase gene; this gene is expressed in a wide variety of gram negative species, suggesting that a fundamental difference exists in gene expression between Enterobacteriaceae and Campylobacter species. Finally, the authors demonstrated the presence of a Campylobacter-specific modification system which modifies residues in the EcoRI restriction site.

Despite these apparent hinderances to the cloning of Campylobacter DNA into E.coli species, there are a few reports of successful cloning experiments. Taylor (1985) successfully cloned the tetracycline resistance determinant from a C.jejuni plasmid into pUC8 and obtained tetracycline resistance in E.coli host cells. Ouelette et al (1987) also cloned a kanamycin resistance determinant from Campylobacter species into E.coli and found this gene to be almost identical to that of transposon Tn903 from E.coli; this indicates that Campylobacter species could act as recipients

for genes originating in members of the Enterobacteriaceae family. As was mentioned earlier (section 1.6.3.i), a kanamycin resistance gene from C.coli has been shown to be identical to the kanamycin resistance gene in gram positive organisms (Lambert et al 1985). It may be prudent then, to consider cloning Campylobacter genes into gram positive hosts, since there appears to be DNA homology between Campylobacter species and gram positive bacteria.

A number of well described techniques are usually used in the characterization of plasmids and these include: curing; conjugation; and transformation. In curing experiments chemical or physical agents are used which interfere with plasmid replication or inhibit enzymes of bacterial cells, to produce plasmid-free host strains. If the loss of a plasmid is associated with the loss of a recognisable phenotypic property, this suggests that the plasmid encoded the gene for this property. However, the inability to cure a phenotypic property may simply mean that the plasmid is refractory to the curing agent used, or that it appears to be resistant because it encodes genes vital for cell viability. Successful curing experiments have been done on C.jejuni (Taylor et al 1987).

Conjugation is the transfer of plasmid DNA by cell to cell contact. Tenover et al (1985) investigated the optimal conditions for conjugation between C.coli and C.jejuni and obtained transfer frequencies of 10^{-3} to 10^{-8}

transconjugants per donor cell. Transformation is the uptake of DNA from the surrounding medium by a cell. Some recipient cells are naturally "competent" (able to be transformed), but usually artificial methods are used to induce competence which favours the uptake of plasmid DNA. Until recently, no methods of transformation had been established for Campylobacter species, although there is one report of natural transformation occurring in C.coli (Fraser and Riche 1987). Miller et al (1988) have developed a method for the genetic transformation of intact C.jejuni cells using electroporation. This involves the application of high intensity electric fields of short duration which make biomembranes transiently permeable. It has traditionally been used in mammalian cells and has recently been modified for use on bacterial cells.

Despite these developments, genetic studies on Campylobacter species are far less advanced than those on other enteropathogens (Walker et al 1986). Conjugation has only been demonstrated between Campylobacter species and not to E.coli; mobilization of the C.jejuni chromosome by plasmids in a similar fashion to Hfr formation in E.coli has not been demonstrated, and, until the development of the shuttle vector, no plasmids from E.coli had been transferred into Campylobacter species.

What of the molecular studies on C.pylori? These have started with the analysis of plasmids, but already, many

problems have been encountered. The organism is more fastidious than other Campylobacter species, requiring very specific growth conditions. A reliable plasmid extraction method still needs to be established and the reason for the refractory nature of the plasmids to restriction enzyme digestion has to be confirmed. The results presented in this work suggest the presence of a repetitive element in one of the plasmids. The fragments containing this element have to be cloned and sequenced to establish the nature of the repetitive element. Curing, conjugation and transformation experiments can only be attempted once a selectable marker has been found. In other Campylobacter species, these markers have been antibiotic resistance determinants which are plasmid mediated. Since C.pylori is sensitive to a wide range of antibiotics and the resistance determinants for the few antimicrobial compounds to which it is resistant are usually chromosomally located, this seems unlikely.

In conclusion, there appear to be many hurdles which must be overcome before the molecular basis of the virulence of C.pylori can be established.

APPENDIX 1

ETHANOL PRECIPITATION OF DNA

DNA was precipitated by the addition of 2,5 volumes of absolute ethanol and 1/10 volume of 4M LiCl and chilling at -70^{o}C for 10 minutes or -20^{o}C overnight. This was followed by centrifugation at 4^{o}C for 10 minutes, drying the pellet under a vacuum and resuspension in water.

PHENOL/CHLOROFORM EXTRACTION OF DNA

All phenol extractions of DNA solutions were done by adding an equal volume of phenol and centrifugation for 2 minutes. This was followed by the addition of an equal volume of chloroform/isoamylalcohol (24:1) to remove residual phenol from the DNA solution and centrifugation to separate phases. The upper aqueous phase containing the DNA was recovered and the DNA was precipitated with absolute ethanol.

APPENDIX 2

PURIFICATION OF DNA FROM AGAROSE GELS

A specific plasmid or DNA fragment is sometimes needed as a probe for hybridization or to use as insert DNA in cloning. This plasmid/fragment has to be separated from other plasmids/fragments in the same DNA preparation. Various methods have been described for doing this, in an attempt to obtain maximum DNA recovery. These include electroelution, elution onto DEAE-cellulose, and the two methods described below which were used in this work.

1. Low Gelling Temperature Agarose

This agarose was prepared and used in the same manner as ordinary agarose. After electrophoresis, the desired plasmid/fragment was cut out of the gel and placed in an Eppendorf tube with a maximum volume of TAE buffer. The tube was placed at 70^{o}C until the agarose had melted (about 5 minutes), before 2 phenol/chloroform extractions were done. The DNA was precipitated with absolute ethanol and the DNA pellet was resuspended in water. This method did not recover very much DNA and was not used routinely.

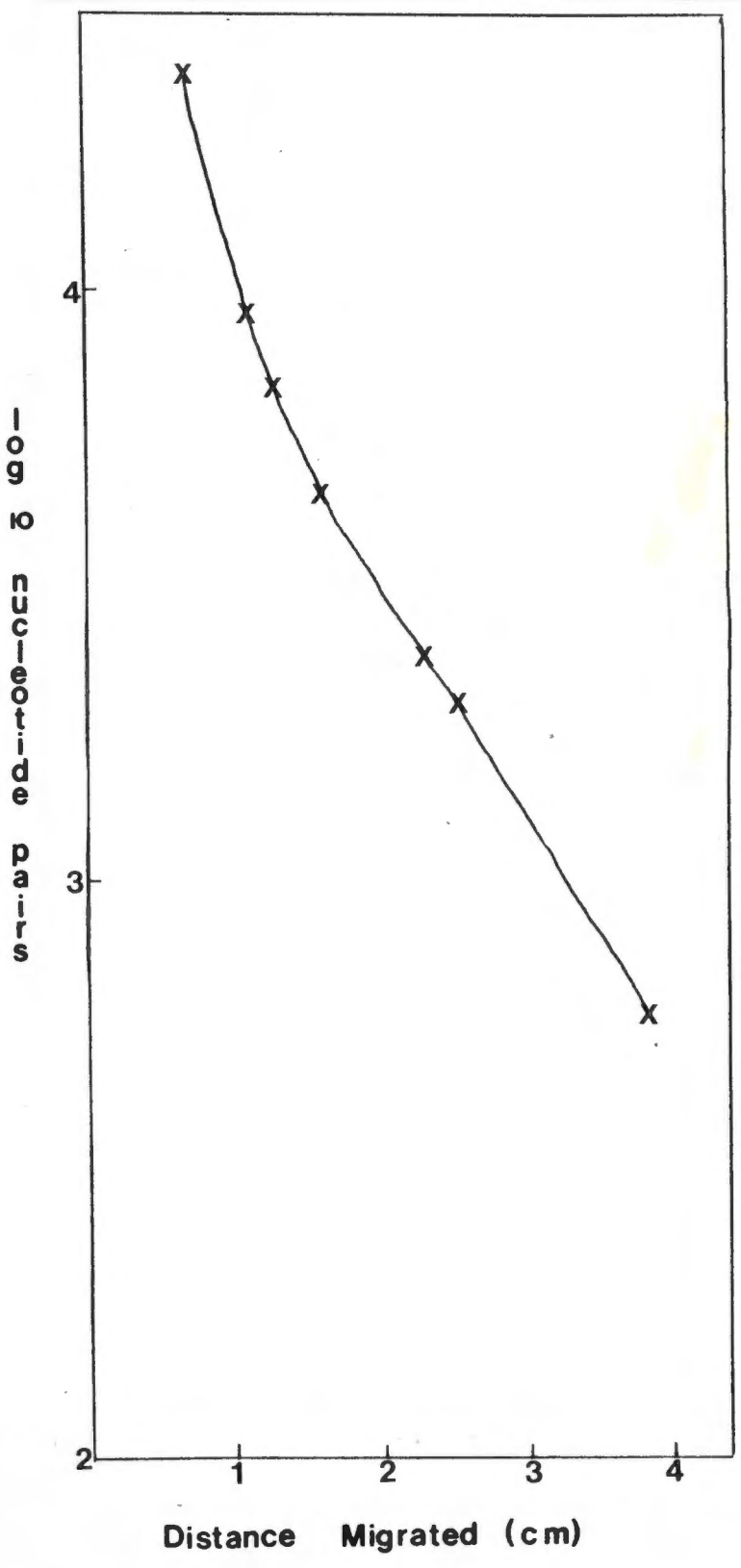
2. Elution of DNA by freezing

After electrophoresis, the plasmid/fragment was cut out of the agarose gel and chopped up very finely with a scalpel

blade. The gel was placed in an Eppendorf with an equal volume of phenol and the tube was placed at -70°C for 20 minutes. This was followed by centrifugation for 10 minutes and recovery of the upper aqueous phase. Two phenol/chloroform extractions were done, followed by precipitation of the DNA with absolute ethanol and resuspension of the pellet in water. This method yielded good recovery of small fragments and was very quick.

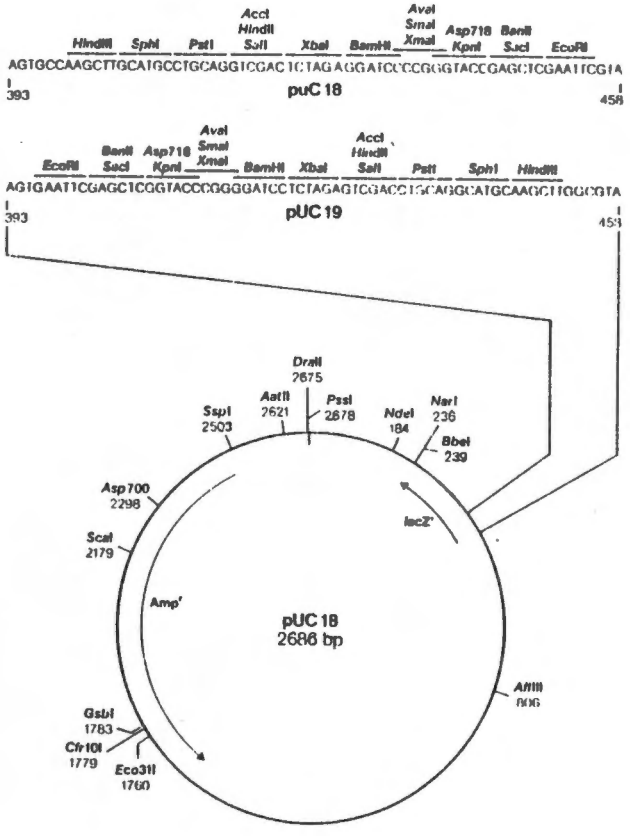
APPENDIX 3

Graph showing the standard curve used to determine DNA fragment sizes.



APPENDIX 4

Map of restriction enzyme sites on pUC 18 and pUC 19.



APPENDIX 5

Publications arising from this thesis:

1) PENFOLD, S. S., LASTOVICA, A. J. and ELISHA, B. G. (1988). Demonstration of plasmids in Campylobacter pylori. Journal of Infectious Diseases, **157**, 850-851.

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Demonstration of Plasmids in *Campylobacter pylori*

COLLEAGUES—The presence of *Campylobacter pylori* in gastric mucosa has been closely associated with histologically confirmed gastritis and peptic ulceration [1]. Presently, nothing is known about the mechanisms of pathogenicity of this organism. Because virulence factors are known to be plasmid mediated in a variety of bacteria [2], we decided to examine isolates of *C. pylori* for plasmids. *C. pylori* strains were isolated and characterized, by using accepted criteria [3], from gastric and duodenal biopsy specimens from patients who had upper-gastrointestinal-tract problems and who underwent gastroscopy at Groote Schuur and Red Cross Children's hospitals, Cape Town, South Africa. Plasmid DNA was extracted by a modification of the method of Lastovica and Ambrosio [4]. Plasmid DNA was detected by electrophoresis in 0.6% horizontal agarose gels run for 18 h at 35 V. Gels were stained with ethidium bromide (1 μg/mL) and visualized at 300 nm.

Twenty (48%) of the 42 isolates examined had plasmids. This is the first demonstration of plasmids in *C. pylori* (figure 1). These plasmids appear to be stable, and most of the isolates showed unique profiles. Plasmids ranged in size from 2.4 megadaltons (MDa) to >96 MDa, but the majority were between 6.6 and 23 MDa. Three isolates from three patients appeared to have identical plasmid profiles, and restriction enzyme digestion with *Bam*HI, *Eco*R1, *Hin*dIII and *Pst*I confirmed that these isolates share a common plasmid of 3.5 MDa. Most isolates had more than one plasmid; however, the multiple plasmid bands present in one isolate were shown by restriction endonuclease analysis to be alternative forms of the same plasmid. Separate biopsy specimens taken from the body and antrum of the stomach of a patient with gastritis yielded two isolates with identical plasmids. Similar biopsy specimens from another patient revealed distinctly different plasmid profiles, a result suggesting a mixed *C. pylori* infection. Plasmid analysis will be useful in characterizing isolates of *C. pylori* in studies of reinfection and in epidemiology. Knowledge of the pathogenicity of *C. pylori* may be greatly enhanced by examining this organism for plasmid-mediated virulence factors.

This work was supported by research grants from the South African Medical Research Council and the University of Cape Town to A. J. L. S. S. P. is a graduate student registered at the University of Cape Town.

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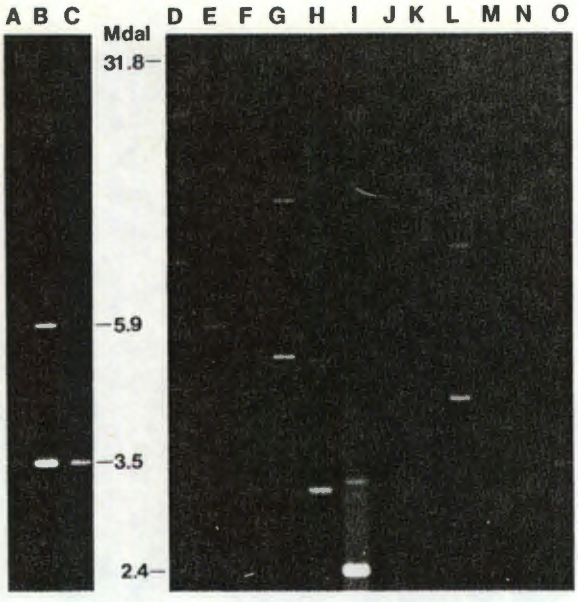

Figure 1. Agarose gel electrophoresis of *C. pylori* plasmid DNA. *Lanes A, B,* and *C* show isolates with similar profiles and a common 3.5-MDa plasmid. *Lanes E* and *F* represent identical profiles of two isolates from the body and antrum of the stomach of a patient with gastritis. *Lanes D* and *G–O* represent unique profiles revealed in other *C. pylori* isolates.

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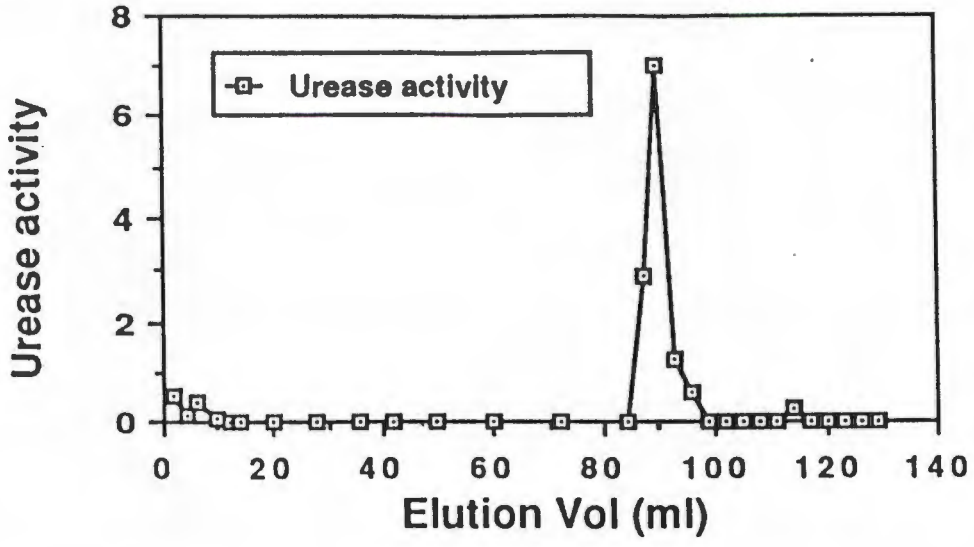

Figure 1.

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Abstract No. 126

Demonstration of plasmids in Campylobacter pylori

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Introduction

Campylobacter pylori has recently been shown by a number of workers to be closely associated with histologically confirmed gastritis, duodenal ulceration and duodenitis. As there is at present no information on the plasmids of *C.pylori*, this present study was undertaken to determine if indeed plasmids are present in *C.pylori*.

Materials and Methods

C.pylori were grown on tryptose blood agar base (Oxoid CM 233) plates with the addition of 5% lysed horse blood. Plates were incubated at 37°C under microaerophilic conditions for 2 to 3 days and harvested with 1.5 ml of STE buffer (0.1 M NaCl, 0.01 M Tris-HCl and lmM EDTA, pH 8.0). Plasmid

extraction was initially performed by the method of Birnboim & Doly (1). However this procedure was found to yield inconsistent results and the method described by Lastovica and Ambrosio (2) was used. A modification of this method involved the inclusion of a phenol/chloroform extraction step after the precipitation and removal of chromosomal DNA. Electrophoresis was performed on 0.6% or 0.8% gels at 30V for 18 hr. Gels were stained with ethidium bromide (1 mcg/ml) and visualized over an ultraviolet light at a wavelength of 300 nm.

Results and Discussion

To date 42 isolates of *C.pylori* have been examined for plasmids. Twenty isolates (48%) have been shown to contain plasmid DNA at levels detectable by the methods described. It is possible that a more sensitive detection method would reveal more plasmids, as some plasmids were present in very low copy number. Plasmid copy numbers varied considerably (as demonstrated by differing band intensities) not only between isolates but within an isolate. *C.pylori* isolates were shown to contain between one and six plasmids, although it remains to be determined if the multiple bands as visualized on the gels represent discrete plasmids or alternative forms of the same plasmid. Plasmids varied considerably in size, from less than 3.1 megadaltons to greater than 96 megadaltons. The majority of plasmids ranged in size from 6.6 to 23 megadaltons. Most of the *C.pylori* isolates had unique plasmid profiles, however a common 3.5 megadalton plasmid was present in three isolates. Separate biopsies taken from the body and antrum of the stomach of the same patient at a gastroscopy session yielded 2 isolates of *C.pylori* with identical plasmid profiles. However, similar biopsies from a second patient revealed different plasmid profiles for the 2 *C.pylori* strains isolated. The possibility of mixed infection exists in this patient. To date we have not been able to assign any function to these plasmids.

Conclusion

Our observations indicate that *Campylobacter pylori* carry plasmids. As no typing system exists for the differentiation of individual isolates of *C.pylori*, plasmid analysis will be extremely useful in examinating patient reinfection and epidemiological studies. Plasmids are well known to mediate a constellation of virulence factors. Continuing research on the plasmids of *C.pylori* will lead to a better understanding of the pathogenicity of this medically important bacterium.

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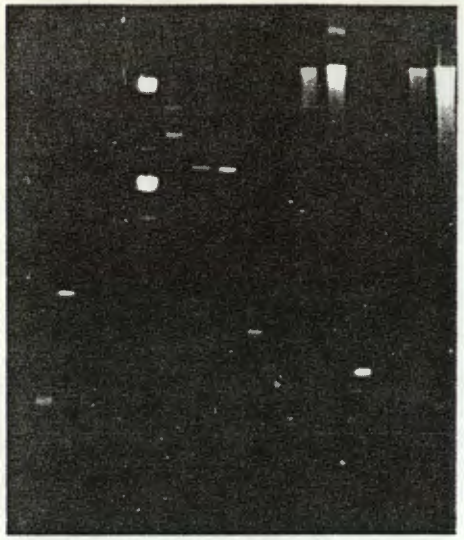

Figure. A typical gel showing a variety of plasmid profiles found in *C.pylori*.

Abstract No. 1

On the occurrence of Campylobacter pylori in patie with chronic gastritis. - Light microscopical and ele tron microscopical studies

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Introduction

Reports from Australia, England, and other countries have indi an association may exist between *Campylobacter pylori* and h gastritis. The aim of the present work was to find how often the occurs in Norwegian patients with gastritis. We also wanted to ex possible effects of the bacteria on the gastric mucosa.

Materials and Methods

Sixty-one patients were included in the study. There were 34 me women. The median age was 53 years (range 16-78). The patients referred to gastroscopy for various reasons, mainly because of up minal discomfort. At endoscopy, 40 patients presented signs compa

CONCLUSION: Constipated LI patients appear more symptomatic during a HBT than LI patients with normal or loose stools. Abdominal distention is the most frequent symptom during the HBT with diarrhoea being less common in both groups of patients.

This study suggests that delayed intestinal motility plays a major role in producing symptoms in LI patients.

Transient Protein Losing Enteropathy Associated with Acute Gastritis and Campylobacter Pyloridis in Children

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Campylobacter like organisms have been frequently reported with active chronic gastritis and gastric and duodenal ulcers in adults. Recently this association has been described in children with symptomatic gastritis. As these organisms are rarely observed with a normal gastric mucosa it has been suggested that they are the cause of the inflammatory change.

This report described 3 children aged between 2.25 and 4.5 years who presented with generalised oedema and hypoproteinaemia due to an acute severe protein losing enteropathy. At gastroscopy, they had features of acute gastritis involving the body and fundus of the stomach. In each the duodenum was normal. Histology of the gastric mucosa confirmed the gastritis and Warthin-Starry staining demonstrated numerous Campylobacter like organisms on the surface of the gastric mucosa. These were subsequently identified as being C.pyloridis by means of electron microscopy and culture of biopsy specimens. Recovery was accompanied by resolution of the gastritis and concommitant disappearance of the C.pyloridis. In 2 cases recovery was dramatic following treatment with erythromycin. In one case no antibiotic was administered and recovery was much slower.

The findings suggest that Campylobacter were responsible for the gastritis and the protein losing enteropathy. This association has not been described before. The diagnosis should be considered in all children with unexplained acute protein losing enteropathy as the organisms are easily identified on histology and the condition is amenable to treatment with antibiotics.

Total Serum Amylase and Isoamylases following Endoscopic Retrograde Cholangiopancreatography (ERCP)

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It has been reported that up to 70% of patients develop hyperamylasaemia following successful visualisation of pancreatic duct (PD) with ERCP.

The aim of this study was to (i) evaluate the pancreatic (Pi) and salivary (Si) isoamylase after ERCP, and (ii) the predictive value of Pi prior to doing ERCP.

PATIENTS AND METHODS: Blood samples were taken from 21 patients before and approximately 4 hours after ERCP. Indications for ERCP were: chronic pancreatitis, obstructive jaundice and chronic abdominal pain of unknown aetiology. Total serum amylase, pancreatic and salivary isoamylases were measured using the Phadebas amylase kit and CAM electrophoresis respectively.

RESULTS: All 21 patients had their PD visualized. 18 pts (85.7%) showed a rise of total amylase above basal level (>30%). In 8 (44%) patients, the rise was due to an increase in Si only. In the remaining 10 patients 9 had an increase in both Si and Pi. Only 1 was due to Pi alone. Of 10 patients with normal Pi before ERCP, 6 had raised Pi above normal after ERCP. These were patients who were shown to have normal or obstructed PD. The other 4 had advanced ductal changes (dilatation). In 4 patients with less than normal Pi before ERCP none had an increase of Pi after the procedure. All had chronic calcific pancreatitis.

CONCLUSIONS: (i) Most patients had hyperamylasaemia following ERCP. It is, however, significant that in 44% of pts the raised amylase is salivary isoamylase and not arising from the pancreas. (ii) Pancreatic isoamylase done prior to ERCP probably has a predictive value in assessing the potential risk of pancreatic hyperamylasaemia with subsequent acute pancreatitis.

The Purification of Pancreatic Enzymes from Duodenal Juice

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Aspirated duodenal juice obtained during hormonal stimulation of the normal pancreas is predominantly pancreatic juice. Consequently, the synthesis rates of protein contained in aspirated juice from patients without pancreatic disease provide reasonably accurate measurements of pancreatic enzyme synthesis rates in man. However, in pancreatic failure, the proportion of juice protein derived from non-pancreatic sources is considerable. Since the turnover rates of such proteins are longer, the mean turnover rate will fall. It is thus necessary to isolate the individual enzymes from the juice. To achieve this two methods have been used.

METHODS: a) Amylase has been isolated from the juice by affinity chromatography using acarbose, an inhibitor of the enzyme, as the ligand, b) Ammonium sulphate fractionation of the juice has been performed and a precipitate containing a mixture of trypsin and chymotrypsin obtained.

RESULTS: Polyacrylamide gel electrophoresis of the purified amylase obtained from method (a) produces a single band. Yields were, however, insufficient to measure specific activity and quantities will have to be increased before synthesis rates can be measured. However, method (b) provided sufficient purified enzyme for the calculation of specific activity of trypsin and chymotrypsin. In one patient with chronic calcific pancreatitis the specific activity was shown to be 1.2 times greater than that of the total juice protein (i.e. unpurified), indicating that 20% of the juice protein (i.e. the non-enzyme protein) had been removed.

These two techniques make it now possible to measure the in vivo synthesis rates of individual pancreatic enzymes in patients with pancreatic disease.

Campylobacter Pyloridis and the Gastric Mucosa

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Campylobacter pyloridis has been found on the gastric mucosa in both normal subjects and in patients with various mucosal diseases. This study was undertaken to determine the frequency of this association in patients undergoing gastroscopy and to investigate the adherence of the bacteria to the mucosa. Biopsies were taken under direct endoscopic vision and placed on sterile blood agar plates or in sterile containers. The biopsies were then rubbed over plates of tryptose blood agar containing 6% v/v unlysed horse blood with swabs dipped in tryptic soy broth and incubated microaerophilicly for 6 days at 37^0. Biopsies were also prepared for electron microscopy.

Biopsies were taken from 51 patients (24 with a gastric ulcer, 6 with a duodenal ulcer, 12 with gastritis and 7 with normal mucosa).

Campylobacter pyloridis was cultered in 73% of the patients. There was no difference in the culture rates in the different groups (gastric ulcer 17/24, duodenal ulcer 6/6, gastritis 9/12 and normal mucosa 5/7). Concomitant therapy with H_2 antagonists did not appear to influence the isolation rate. On electron microscopy the bacteria were attached by their flagella and penetrated the mucosal cells.

As the carrier rate of campylobacter pyloridis appears similar in patients with a variety of upper gastrointestinal conditions, it's clinical significance appears uncertain. Penetration of the mucosal cells by bacterial flagella may be of importance in enabling the bacteria to survive the hostile gastric environment.

The Relationship between Hepatocullular Carcinoma and Hepatitis B Virus at Ga-Rankuwa Hospital, Pretoria

IR BASETE, JJ ALEXANDER CF VAN DER MERWE. Division of Virology, Department Microbiology, Department Medicine, Medical University of Southern Africa

Blood samples of 59 histologically proven hepatocullular carcinoma (HCC) patients were tested for hepatitis B (HB) markers (HB surface Antigen (HBsAg), HB surface Antibody (HBsAb) and HB core Antibody (HBcAb)). All HBsAg positive serums were further tested for HB e Antigen (HBeAg) and HB e Antibody (HBeAb). The data from these patients were compared with those obtained from a group of 31 age- and sex matched control patients. Forty-nine HCC cases (83%) showed serological evidence of exposure to HBV shown by the presence of one or more of the HB markers. Eighteen patients (37%) were positive for HBsAg. Of these 16 were males.

Because of the insufficient volumes of serum, only 12 patients with HBsAg positive serum were tested for HBeAg and HbeAb. Of these cases, 3 were negative for both, 7 were HBeAb positive and one HBeAg negative. One patient was positive for both and one HBeAb negative and HBeAg positive.

In the control group 17 were negative for HB virus (55%), 7 cases were HBsAb positive only and one was HBsAg and HBcAb positive.

It is possible that other factors in our area have aetiological significance for HCC as 17% were negative for any marker of HBV.

Essential Fatty Acids and Their Metabolic Intermediates as Cytostatic Agents. The Use of Evening Primrose Oil (Linoleic and Gamma-Linolenic Acid) in Primary Liver Cancer. A Double-Blind Placebo controlled trial.

CF VAN DER MERWE, J BOOYENS. Department of Medicine and Physiology, Medical University of Southern Africa.

The cytostatic effect of the metabolic intermediates of essential fatty acids on various cancer cell lines in vitro, initially demonstrated in our laboratories, has now been confirmed by various other investigators in this field, in various countries. One of the cell types used by us in two different experiments was the Alexander hepatoma cell line. The proliferation of this cell type was consistently inhibited in vitro by gamma-linolenic acid. The complete lack of reported side-effects from this normal dietary constituent and metabolic intermediate of the essential fatty acid, linoleic acid and the dismal prognosis of African primary liver cancer patients, prompted a double blind placebo controlled trial using Evening Primrose Oil (EPO) (60% linoleic acid, 9% gamma-linolenic acid) as a dietary supplement.

A total of 63 patients, all with histologically proven hepatoma, were randomly allocated to either 36 capsules/day of Evening Primrose Oil (containing a total of 1.62g gamma-linolenic acid) or to placebo.

RESULTS: No statistical difference in survival time between the 2 groups could be shown. ($p = 0.26$) The complete absence of side-effects as well as the general symptomatic improvement observed in EPO supplemented patients should be recorded.

It is suggested that higher doses of gamma-linolenic acid, administered intravenously should achieve a more significant effect in this malignancy, since liver tumours often weigh as much as 3 kg.

Precursor Lesions of Oesophageal Carcinoma at Medunsa

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AIM: To detect early changes in a high risk population.

MATERIAL AND METHODS: Patients attending the GE from January 1987 in the age group 40 - 60 years underwent gastroscopy.

Excluded when history of dysphagia, bleeding, endoscopic findings of reflux oesophagitis, stomach Ca., macroscopic OC.

Parameters studied were: tribe, living area, occupation, alcohol-tobacco consumption, endoscopic diagnosis.

Biopsies: (3 each) were taken from the first, middle, lower oesophagus.

RESULTS: 20 patients are collected in this ongoing study of which 15 males, 5 females, similar M/F ratio as our OC group.
Tribe: 40% Tswana, rest mixture of other tribes.
Living area: 60% surrounding urban townships, 40% surrounding rural areas.
Occupation: 40% unemployed, 40% unskilled, 20% skilled.
Alcohol: 75% no alcohol, 25% alcohol,
Smoking: 65% no smoking, 35% smoking.
Diagnosis on scope: 60% no abnormality, 25% gastritis, 15% ulcer balbi.
Biopsy results: 20% mild dysplasia, in first and lower part mainly.
CONCLUSION: 20% showed mild dysplasia as early change in a high risk group. Mass surveys like in China might reduce the high mortality rate till the definite cause of OC is found.

A Marker Technique to Evaluate a Simplified Pancreatic Stimulation Test Utilizing Cimetidine

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Contamination of duodenal contents by gastric juice is normally prevented during the standard pancreatic stimulation test by aspirating the stomach separately. Presuming that the same effect can be achieved by utilising cimetidine to suppress gastric acid secretion, and not having to intubate the stomach, we have shown that bicarbonate and enzyme concentrations obtained in this manner compare favourably with values of standard procedures.

The present study was undertaken to estimate the recovery rate of duodenal contents by this method and to establish to what extent duodenogastric reflux and loss of duodenal contents into the jejunum took place.

METHOD: Four normal volunteers were examined by the standard pancreatic stimulation test utilising secretin/CCK. Cimetidine was administered to suppress gastric acid prior to the procedure. A double-lumen duodenal tube was devised making it possible to infuse an isotope marker (tin colloid) into the upper part of the duodenum at a constant rate. The other tube was positioned at the junction of the 2nd and 3rd parts of the duodenum, or slightly more distally, and pancreatic secretion was aspirated through this by several openings. The amount of radioactivity infused was compared to that which was recovered and expressed as a percentage. The duodenal aspirate was also analysed for bicarbonate, amylase and chymotrypsin concentration.

RESULTS: The recovery rate of duodenal juice varied between 74% - 86%. Duodenogastric reflux could be demonstrated to account for 4 - 10% and loss into the small bowel was only 2 - 6%.

CONCLUSION: Recovery of duodenal contents by this method is satisfactory and it can be recommended for clinical application. Loss of duodenal contents into the jejunum or by reflux into the stomach appears to be insignificant.

Disassociation of Hepatic Function as Estimated by Liver Imaging

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Comments

Dr Szabo: Dr Hinder, you presented some nice data on free radicals which represent one of the biochemical tools for vascular injury and epithelial damage. You showed effects with a new compound conjugating superoxide dismutase (SOD) with arginine to prolong its half-life. Is this the case?

Dr Hinder: Yes, though it was a biochemist, Thys Oosthuizen, in Johannesburg who did this. He conjugated albumin with SOD, and prolonged the half-life to 60 hours.

Dr Szabo: Can you give this orally or do you still have to administer it intravascularly?

Dr Hinder: It has to be given intravascularly.

Dr Szabo: Did you try to use catalase in these studies? In our studies on chemical-induced lesions, catalase was almost equi-active with SOD.

Dr Hinder: Most of the literature suggests that catalase is as good as SOD. We did not test it but I would have expected the same result with catalase.

Dr Szabo: Although there are several studies showing that free radical scavengers are important, especially in stress-induced lesions, biochemically there is no evidence of lipid peroxidation in the gastric mucosa. We studied four biochemical parameters of lipid peroxidation and found no evidence of lipid peroxidation under the same conditions that SOD and catalase are active. The conclusion must be that there is some other target that these free radicals are hitting other than peroxidation of lipid. Do you have any idea about that?

Dr Hinder: There are a lot of theories in this area which do not really hold up when tested. We have both shown this. My feeling is that the mechanism is still very murky. There is still a lot of work to be done.

Campylobacter pylori and the gastric mucosa

J. P. WRIGHT, A. J. LASTOVICA, M. EMMS, S. S. PENFILD

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The recently recognised association between *Campylobacter pylori* and gastric ulcer and gastritis has raised the question of an infective cause of peptic ulcer disease. We have been looking at the relationship between the *Campylobacter* organism and the gastric mucosa in endoscopic biopsies. The *C. pylori* is a micro-aerophilic organism with flagella normally arranged on one end of the cell, although some organisms have two sets of flagella, one at each end. It is interesting that, on looking back at old histological specimens, we found that *Campylobacter* were present but were missed when the specimens were originally examined.

The *C. pylori* are arranged on the tight junctions, or in the mucus lining the gastric mucosa (Fig. 1). This predilection for tight junctions is common in our specimens, but the reason for this is not clear. It may be due to the presence of preferred metabolites or growth factors such as haemin and urea at the tight junctions since all *C. pylori* isolates have an extremely high urease activity and require haemin for growth. There may also be some effect of these bacteria on the cells, since associated dying cells with bacteria arranged along the tight junctions and in the microvilli of the cananiculi of the dying cell were quite frequently seen (Fig. 2). The presence of these bacteria on the tight junctions appears to have an effect on the adherence of the cells as there appears to be a splitting and opening up of the tight junctions followed by crenation and death of the mucosal cell. *Campylobacter* appears to adhere to these cells by means of their flagella. Further evidence of this adherence is the thickening of the bacterial wall alongside the gastric mucosa.

The *C. pylori* also appear to penetrate deep into the mucosa via the glands, and so on cross-section are seen quite deep in the gastric mucosa. It is noteworthy that there is very little inflammatory action around these deeply penetrating organisms. However, where the cells are dying, there is an influx of inflammatory cells which are presumably moving to the dying mucosal cell rather than the *Campylobacter* itself.

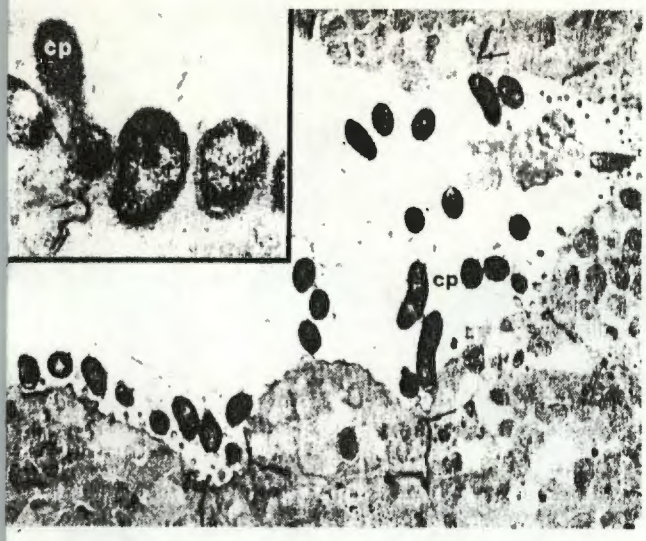

Fig. 1. Low-power electron micrograph showing *C. pylori* (CP) clustered at the intercellular tight junctions (arrows). Inset: Higher magnification of *C. pylori* (CP) wedged into a tight junction. Adjacent organisms have indented the cell membrane.

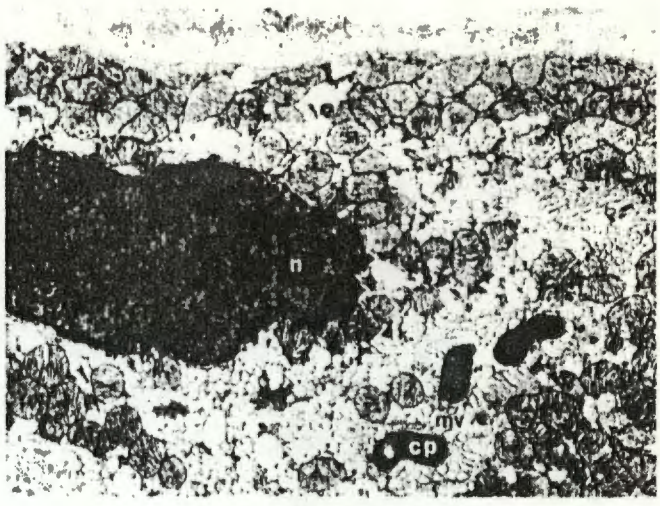

Fig. 2. Low-power electron micrograph showing *C. pylori* (CP) which have penetrated into the microvilli (MV)-lined cananiculi of a necrosing parietal cell with nuclear pyknosis (n).

At present, nothing is known about the mechanisms of pathogenicity of *C. pylori*. Since virulence factors including those involving adherence are known to be plasmid-mediated in a variety of bacteria, we examined 48 *C. pylori* isolates and found that 26 (54%) carried unique plasmid profiles. Continuing research on the plasmids of *C. pylori* may lead to a greatly enhanced knowledge of pathogenicity in this organism.

In summary, we have demonstrated adherence of the *Campylobacter* to the gastric mucosal cells, possibly by the flagella. We have also demonstrated crenation and death of these cells in association with collections of bacteria on the tight junctions.

Campylobacter pylori

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Campylobacter pylori are micro-aerophilic, Gram-negative, oxidase- and catalase-positive bacteria.[1] One of the remarkable properties of this organism is its great ability to split urea.[2]

Methods of diagnosis of *C. pylori*

Antral biopsy

Histology and culture

C. pylori can be detected histologically on the mucosal surface of most biopsy specimens showing active chronic gastritis.[3] The bacteria are faintly visible on conventional haematoxylin and eosin sections, but the Warthin-Starry silver stain is said to show the organisms more reliably.[4] This method is expensive and time-consuming. A modified Giemsa stain (without differentiation) is quick, simple and appears to be as reliable as the Warthin-Starry stain.[3] All of these methods are nonspecific and will label any bacteria present in the stomach. The characteristic curved morphology of *C. pylori* and its close association with the enterocyte surface, however, allows presumptive histological diagnosis in almost all patients.

In our experience, the conventional haematoxylin and eosin stain proved to be very effective, as it was able to pick up all cases which were shown by the Warthin-Starry silver stain technique to have *C. pylori*.

Antral biopsies are plated on chocolate agar and incubated micro-aerophilically at 37°C for up to 10 days. Suspect colonies are identified as *C. pylori* by the oxidase test, a positive catalase test and a characteristic rapid urease reaction.

Rapid methods for detection of urease

The remarkable property of *C. pylori* to split urea is the basis of two tests of urease activity: (*i*) a biopsy specimen is placed in Christensen's urea broth and examined frequently for any colour change from yellow to pink or orange (positive). After 24 hours, the test can be considered negative if no colour change has occurred. (*ii*) CLO Test (Delta West Ltd, Perth, Australia). In this test, the same procedure as in subsection (*i*) is followed.[5] The test uses an agar medium. This appears to be a more sensitive test than the use of Christensen's urea test, in that in one study,[5] the test was positive in 79% of cases within 1 hour, compared with 56% of cases using Christensen's urea test.

Methods not using antral biopsy

Serum antibodies.[6] In this study, 16/19 patients who were *C. pylori*-positive were positive by the ELISA test

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OFFICIAL ABSTRACT FORM

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Start→ Characterisation of Plasmids In Campylobacter pylori

PENFOLD SS[1], LASTOVICA AJ* and STEYN LM[1]. University of Cape Town[1] and Red Cross Children's Hospital, Cape Town, South Africa.

Characterisation by restriction enzyme mapping, of plasmids isolated from C.pylori, was undertaken.

To construct a restriction map of a 6.2kb plasmid found in 5 isolates, DNA isolated by the method of Birnboim and Doly was digested by a number of enzymes under appropriate conditions.

The majority of the restriction enzymes used did not digest C.pylori DNA. MboI did not cleave the DNA, whereas digestion with its isoschizomer, Sau3A, resulted in two large fragments of 2,4 and 2,3kb in size as well as several smaller fragments.

The DNA of C.pylori appears to be methylated since it is not digested completely by enzymes sensitive to methylation of the substrate. In addition, the presence of two large fragments of 2,4 and 2,3kb after digestion with Sau3A rather than a number of ±256 base pair fragments, which one would expect, suggests that this DNA contains repeated sequences.

Telex:

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Signature

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