MOLECULAR TECHNIQUES FOR THE STUDY OF *HELICOBACTER* SP. INTERACTION WITH HOST MICROFLORA IN THE MOUSE MODEL

ENG

by

Ramahi Sarma-Rupavtarm

B. Tech. (Hons) (Biotechnology and biochemical engineering)

Indian Institute of Technology, Kharagpur, 1998

Submitted to the Department of Civil and Environmental Engineering

in partial fulfillment of the requirements for the degree of

Master of Science in Civil and Environmental Engineering

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

June 2000

Author .....

Department of Civil and Environmental Engineering
May 19, 2000

Certified by .....

Martin F. Polz
Assistant Professor
Thesis Supervisor

Accepted by .....

Daniele Veneziano
Chairman, Departmental Committee on Graduate Studies

Molecular techniques for the study of *Helicobacter* sp. interaction with host microflora in the mouse model

by

RAMAHI SARMA-RUPAVTARM

Submitted to the Department of Civil and Environmental Engineering
on May 19, 2000 in partial fulfillment of the
requirements for the Degree of Master of Science in
Civil and Environmental Engineering

ABSTRACT

Helicobacter spp. are known animal pathogens. Infection by *Helicobacter pylori* in humans leads to gastritis and ulcers and has been implicated as a causative agent for gastric cancer. Animal models are useful for studying pathogenesis and host-pathogen interactions for pathogens like *H. pylori*. One such model is the mouse model colonized with a defined group of eight organisms representative of the normal mouse intestinal flora, the altered Schaedler flora (ASF). Infection by a pathogen may lead to changes in the populations of the host microflora, which are difficult to study by conventional culture and biochemical methods. The aim of my work is to develop PCR-based techniques to study the interaction of *H. pylori* with the ASF microflora in mice. Primers targeting the 16S rRNA gene for the detection of *H. pylori* and *Helicobacter* genus bacteria by PCR were developed. The primer pair, 787f -1127r, was evaluated and was found to be specific for *H. pylori* and was able to detect 40 copies of the gene in a background of mice fecal pellet DNA. Genus specific primer pairs 212f-289r and 212f-1026r were able to detect all the helicobacters tested, but also amplified DNA from other organisms and are being redesigned.

Two methods, terminal restriction fragment length polymorphism (t-RFLP) and constant denaturant capillary electrophoresis (CDCE), were assessed for studying the diversity of the ASF species. T-RFLP is based on differences in lengths of terminal fragments obtained by restriction digestion of the amplified 16S rRNA gene from a mixture of different organisms. CDCE separates amplified fragments of the 16S rRNA gene on the basis of melting temperature differences due to sequence variation between different microbes. An artificial mixture of 4 ASF bacteria could be analyzed successfully by T-RFLP. CDCE analysis led to anomalous results due to the use of degenerate primers. The method was, however, very sensitive and is being retried with a different set of primers. These techniques provide a sensitive and accurate means for studying the diversity of ASF bacteria in response to insults like pathogens. Future work is aimed at using these techniques for pathogen ecology research in ASF mice.

Thesis supervisor: Martin F. Polz

Title: Assistant professor of Civil and Environmental Engineering

Acknowledgements

Thanks first and foremost to Professor Martin Polz for his guidance and support, to Professor David Schauer for his guidance and help in getting all the DNA samples, to Professor Bill Thilly for letting me use the CDCE instrumentation, to my labmates Vanja, EeLin, Aoy, Linping, Janelle, Stefan and Cathy for their encouragement and help, to Ryan for all the GeneScan help, to Dr. Bruce Paster of the Forsyth Institute for kindly helping me out with DNA samples, and last but not least, to my family and all my friends.

Table of contents

List of figures and tables

Chapter 1

Helicobacter pylori and the ASF mouse model : an introduction

History and taxonomy

Even in the late nineteenth century, European pathologists had observed curved bacteria in the gastric tissues. However, they were unable to culture them and their work went unnoticed for a long time. In 1979, Robin Warren, an Australian pathologist noticed curved bacteria in gastric biopsy specimens submitted for histological examination. Together with Barry Marshall, he noticed that the bacteria were curved Gram negative rods with morphological similarity to the *Campylobacter* species (Marshall *et al*, 1984). Consequently, he used methods for isolation of bacteria of the *Campylobacter* species. The cultures did not grow over the three day period characteristic of campylobacters but one culture accidentally left over a weekend showed growth (Marshall *et al*, 1984).

The organism was characterized and called *Campylobacter pylori*. However, cellular fatty acid profiles showed fatty acid types not present in campylobacters. One of the fatty acids found was unique and the overall novelty of the fatty acid profile led to a new name and genus designation, *Helicobacter pylori* (Goodwin *et al*, 1985). Bacteriological and molecular studies in conjunction with 16S rRNA gene sequence analysis revealed that there were many differences between *Helicobacter pylori* and the *Campylobacter*, *Flexispira* and *Wolinella* species which are closely related phylogenetically. Based upon these studies, it was postulated that *H. pylori* was in a genus separate from campylobacters and was more closely related to *Wolinella succinogenes* (Romaniuk *et al*, 1987, Paster *et al*, 1988).

Several other organisms of the *Helicobacter* genus have since been identified. The *Helicobacter* genus has 17 species altogether out of which four have been found in humans, *H. pylori*, *H. fenneliae*, *H. cinaedi* and *H. heilmanii* (**Table 1.1**). Many of these species have not been cultured and some have tentative names. More and more helicobacters are being discovered with ongoing efforts in this field.

Table 1.1: *Helicobacter* species known currently

Species	Source
Helicobacter pylori	human stomach
Helicobacter mustelae	ferret stomach
Helicobacter felis	dogs and cats
Helicobacter bizzozeroni	dog stomach; looks like "heilmannii"
Helicobacter salomonis	dog stomach; looks like "heilmannii"
Helicobacter acinonyx	cheetah stomach
Helicobacter nemestrinae	stomach of pig-tailed macaque monkey
Helicobacter "heilmanii" [1]	widely distributed group
Helicobacter "suncus"	stomach of tree shrew
Helicobacter muridarum	mouse intestine
Helicobacter canis	dog lower bowel and liver
Helicobacter cinaedi	humans
Helicobacter fennelliae	humans
Helicobacter pullorum	chicken intestine and liver
Helicobacter pametensis	bird feces (terns)
Helicobacter hepaticus	important mouse pathogen in intestine and liver
Helicobacter bilis	rat intestine
Helicobacter trogontum	hamster intestine
Helicobacter cholecystus	mouse intestine
Helicobacter rodentium	blood of AIDS patients; not formally named
Helicobacter "mainz" [2]	blood of immunocompromised patients
Helicobacter "westmeadii" [2]	blood of immunocompromised patients
Helicobacter "colifelis" [1]	cat
Helicobacter "rappini" [3]	sheep, mice, dogs, humans

(Source: Dr. D. Schauer, MIT, 1999)

[1] not cultured; morphologic basis
[2] not formally named
[3] probably more than 1 species

Morphology and pathology

H. pylori organisms are spiral, microaerophilic gram negative bacteria that demonstrate bluntly rounded ends in gastric biopsy specimens.(Dunn *et al*, 1997) They are in the size range of 2.5 to 5.0μm in length and 0.5 to 1.0 μm in width (Figure 1.1). The periodicity of the spiral is 1 to 2μm. It's surfaces are smooth and it has four to six sheathed flagellae emerging from one of its rounded ends. Prolonged culture on solid or liquid medium leads to predominantly coccoid forms. These coccoid forms are in the viable but non-culturable (VBNC) state.

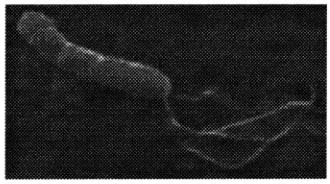

Figure 1.1: TEM image of *Helicobacter pylori*

(Source: www.canadianhp.com)

H. pylori infection can be active or asymptomatic. When active, it has several clinical manifestations. The chief among these are chronic gastritis (an inflammatory response to the infection), non-malignant gastric ulcers, duodenitis, gastric lymphoma and carcinoma. The International Agency for Research on Cancer announced in 1994 that "*H. pylori* plays a causal role in the chain of events leading to cancer." Carriers of *H. pylori* are at a greater risk of atrophic gastritis, which is a precursor lesion to gastric cancer.

Infection with *H. pylori* results in the colonization of the non-acid secreting mucosa of the stomach and leads to an inflammatory response resulting in chronic gastritis in many individuals. This was proved by two healthy volunteers who ingested *H. pylori* and developed chronic gastritis (Marshall *et al*, 1985). Another physiological

effect of infection is the development of peptic ulcer disease. An ulcer is defined as a breach of the mucosa of the alimentary tract. *H. pylori* infection has been seen in nearly all patients with duodenal ulcers and 70% of those with gastric ulcers. An ulcer is thought to develop as a consequence of an imbalance between the gastroduodenal mucosal defense forces and the damaging forces. However, only 10-20% of all people infected with *H. pylori* actually develop a peptic ulcer.

As mentioned before, *H. pylori* infection is seen to be one of the many factors that increase the risk of gastric cancers. The reason for this is thought to be the tissue repair processes that follow chronic gastritis symptoms resulting in metaplasia (change of tissue type during regeneration) and neoplasia (generation of immortal cells) (Cotran *et al*, 1999).

Pathogenesis

Once the host is colonized with *H. pylori*, infection persists for life unless antimicrobial therapy is administered. The highly acidic nature of the stomach makes this a relatively difficult niche to colonize. While other bacteria are present in the digestive tract, only *H. pylori* infects the acidic regions of the tract. The presence and persistence of infection indicates that *H. pylori* is specifically adapted to occupy this niche environment. A key factor contributing to the ability of *H. pylori* to survive in the hostile environs of the stomach is its ability to produce urease (Smoot *et al*, 1990). The bacterium utilizes the urease to break down the urea in the surroundings (present at 1mM concentrations) to form OH^- ions which neutralize the immediate environs of the

bacterium. The production of OH^- ions and ammonia, however, is toxic to the gastric epithelial cells. (see reactions)

$$C{=}O(NH_2)_2 + H^+ + 2H_2O \xrightarrow[\text{Urease}]{} HCO^{3-} + 2(NH_4^+)$$

$$H_2O + HCO_3^- \rightarrow H_2CO_3 + OH^-$$

Reactions for conversion of urea to ammonia and hydroxyl ion

Other important factors that assist the pathogenicity of the organism are its spiral shape and the motility of its flagellae. The spiral shape is especially important in viscous fluids like the mucous layer overlaying the gastric epithelium, as it makes motility in the mucus easier than with a rod shape. These factors make *H. pylori* resistant to peristaltic flushing of the contents of the gastric mucosa and help it persist in the mucosal layer. The latter function is also aided by the production of various adhesin proteins which help *H. pylori* bind to mucosal and epithelial cells.

Virulence factors

A major cause of virulence in *H. pylori* is the vacuolating cytotoxin, which induces vacuolation of epithelial cells which causes injury of the gastric epithelium. The size of this protein is about 87 kDa and it is a product of the polymorphic *vacA* gene, which is about 3.9kb in size (Cover *et al*, 1994). This gene is present in all *H. pylori* strains, but is active only in Type I strains, which are defined by the presence of an active *vacA* gene. Type II strains do not produce functional VacA toxin and have been seen to

cause only mild gastritis in the mouse model. The activation of the *vacA* gene is by a factor known as cagA (cytotoxin activating gene). This locus is present on a pathogenicity island and hence is thought to control the expression of vacA.

While the *vacA* gene is present in all *H. pylori* strains, the cytotoxin is produced only by Type I strains. It was initially thought that lack of production of the toxin was due to sequence divergence between strains, with the negative strains having a 65% nucleotide sequence identity at the *vacA* locus with the positive or Type I strains. This argument is not valid as it has recently been shown that the gene is transcribed in all strains. However, the levels of transcription are different in the positive and negative strains. The *vacA* gene locus has conserved regions and highly divergent regions recurring within the alleles (mosaicism). The variable sequences are of two types, signal sequences and mid-regions. Different combination of these two regions lead to differing toxin production levels. The pathogenicity island within which the *cagA* locus lies codes for a type IV secretion system. This is similar to the system that is involved in the conjugative transfer of DNA in *E.coli* (Covacci *et al*, 2000).

Another factor that has been implicated in the pathogenic effects of *H. pylori* is the molecular mimicry exhibited by its surface antigens. They are postulated to trigger an autoimmune response against uninfected gastric mucosal cells bearing antigens which are recognized by the antibodies raised in response to *H. pylori* surface antigens.

Epidemiology and transmission

H. pylori is present in more than half of the world's population. About 70-90% of the developing countries' population has *H. pylori* infection while 25-50% of the developed world is infected. Most of the infection is acquired during childhood. However, a majority of the infected population shows no symptoms. Other than the human stomach, there are few other known reservoirs of *Helicobacter pylori.* Old World macaques were found to be colonized with *H. pylori*, but they are not a source of infection to humans. There have been studies on *H. pylori* infection in cats (Handt *et al*, 1995) but the exact source of infection to the cats is ambiguous and may have been human.

Thus, one of the major questions is the mode of transmission of *H. pylori* from one person to another. There seem to be three routes for this. The first is iatrogenic where the bacterium is transferred via the endoscope. With better sterilization measures, this is not a major factor now. The second mode is fecal-oral. However, *H. pylori* is rarely shed fecally. A study showed that *H. pylori* was present in the drinking water sources in Peru and may be the source of infection to humans there. (Hulten *et al*, 1996) The third mode of transmission is oral-oral which is seen in Africa, where mothers premasticate food before feeding it to infants.

Genome

The sequencing of the complete *H. pylori* genome has led to an increased understanding of the pathogen (Tomb *et al*, 1997). This knowledge helps in developing probes and primers to different genes and the identification of new therapeutic targets that are unique to *H. pylori.* Two different strains of *H. pylori* (strain J99 isolated in the

USA and strain 26695 isolated in the UK) have been sequenced and compared (Alm *et al*, 1999). The genome size of *H. pylori* ranged from 1.6 to 1.73Mb for the two strains studied. The small size of the genome is consistent with that of other bacteria specialized to living in a single environment. The mechanisms for environmental adaptation such as two component regulatory systems are rare. The bacterium has 4 known two-component regulatory systems as compared to 90 for *Pseudomonas aeruginosa*, an organism that can survive in many different environments (Covacci *et al*, 2000). A study that examined the allelic variation in 6 genes by multilocus enzyme electrophoresis shows a high level of diversity among different isolates (Go *et al*, 1996).

A comparison of the whole genomes of the two sequenced strains, however, showed that the diversity was mostly due to molecular reorganization events and not due to vast differences in genes between the strains (Alm, 1999, Doig *et al*, 1999). A study of the fingerprint of the organism from a single patient over a long period of time did not show much variation, indicating that mixed infections are uncommon (Miehlke *et al*, 1999). However, there are several factors like nucleotide mutations, excision of the pathogenicity island, transposition of insertion elements, recombination with non-colonizing strains and horizontal transfer of new genes that may lead to the kind of microdiversity that can be seen by single nucleotide polmorphism analysis or other sensitive methods. (Covacci, 2000)

Diagnostic tests for *H. pylori*

Both direct and indirect tests are used to indicate the presence of *H. pylori* in the stomach linings. The direct tests are blood tests and endoscopy tests of the stomach. The

indirect tests are the ^{14}C or ^{13}C urea breath tests. For clinical purposes, the tests are divided into invasive and non-invasive tests. Endoscopy tests are invasive.

Invasive tests

Endoscopy of the stomach

A narrow flexible tube is inserted into the oesophagus. The oesophagus, stomach and duodenum are studied through this tube. Several biopsy samples are taken and sent for analysis by culture methods to determine whether *H. pylori* is present or not. Culture methods have the advantages of allowing antimicrobial susceptibility testing and detailed characterization of the cultured organism. However, culture methods typically take 3-5 days and hence are very slow. Endoscopy samples can also be assessed by histological methods. *H. pylori* can be visualized with hematoxylin and eosin (H&E) staining of tissue sections. There is a possibility of false identification with this stain. Hence, a sensitive staining technique consisting of a combination of H&E staining, Steiner silver staining and alcian blue staining is used for more accurate results (Genta *et al*, 1994). Another application of endoscopy samples is in the tissue urease tests. However, these tests are dependent on the bacterial load in the stomach. Tissue biopsy samples obtained by endosopy can also be used for PCR analysis. The accuracy of diagnosis depends on the quality of primers and DNA preparation.

Non-invasive tests

Blood tests

Infection of the gastric mucosa with *H. pylori* results in immune responses leading to an elevation of serum immunoglobin IgG and IgA levels and IgA and IgM in

the stomach. Blood tests check for the the presence of these antibodies. The presence of antibodies indicates the presence of *H. pylori* currently, or an infection in the past (till three months prior to the test) and thus may give a positive test for the pathogen even after it has been eradicated.

Breath test

The breath test depends on the ability of *H. pylori* to metabolize urea to yield CO_2. Urea is labeled with ^{14}C (or ^{13}C) and the breath of the patient is then tested in 20 minutes after ingestion of urea. The presence of $^{14}CO_2$ (or $^{13}CO_2$) indicates an *H. pylori* infection. The only difference in procedure for the two isotopes is that for ^{13}C, the measurements are made by using mass spectrometry instead of scintillation counters.

Detection methods for *H. pylori*

While several of the diagnostic methods for *H. pylori* are very accurate, they are not sensitive enough to confirm the complete eradication of the organism after treatment. Culture methods are unable to detect the organism once it enters the coccoid VBNC form. Also, positive results in some of the tests like serological tests for antibodies can yield misleading results, as the antibodies persist for some time even after the infection is cleared. Since the presence of an organism in a system can be accurately indicated by its DNA or mRNA expression, these form good indicators for the presence of *H. pylori* in clinical samples.

Animal models in the study of *H. pylori*

H pylori is limited in its ability to infect animals. The best models for the infection are non-human primate models, but these are very costly and ethically problematic. Thus, there have been efforts to develop other animals as models. Barrier-maintained piglets were one of the earliest used models, but they are exceedingly costly to maintain and the risk of contamination is high (Eaton *et al*, 1991). Dogs and cats have also been studied as models (Fox *et al*, 1995), but the cost factor in such studies makes them equally prohibitive. The chief advantage of using larger animals in studies is that repeated endoscopy samples can be taken. Among small animals, Mongolian gerbils are a very promising model as their infection mimics that in humans very well (Hirayama *et al*, 1996).

The most viable model system for the study of *H. pylori* pathogenesis would be infection in mice, as they are easy to grow and maintain, and are relatively inexpensive. However, *H. pylori* does not naturally infect mice and the infection when present does not mimic that in humans. Thus, the first mouse infection of *H. pylori* was in "nude" mice, which are immunodeficient (Karita *et al*, 1991). Since then, many laboratories have been able to infect immunocompetent mice also (Marchetti *et al*, 1995). One of the drawbacks with mice has been that the inflammation is very moderate. However, inbred mice have been reported to show higher degrees of inflammation. Also, infection by *Helicobacter felis* in mice leads to much higher inflammation levels (Mohammadi *et al*, 1996). Thus, while mouse models may not be ideal for studying the disease, they are good models for endpoint studies to detect the presence or absence of infection in response to treatment.

Germ-free mice and the Altered Schaedler Microflora

Because the normal mouse gut is home to a plethora of species with very high levels of complexity, a more convenient and easy to manipulate defined murine microbiota of eight species was proposed by Russell Schaedler in 1965. However, since some of the microbes were extremely oxygen sensitive and very difficult to grow, they were underrepresented in the Schadler microflora The species included in the original Schaedler flora were *E.coli* var. *mutabilis*, *Streptococcus faecalis*, *Lactobacillus acidophilus*, *Lactobacillus salivaris*, group N *Streptococcus*, *Bacteroides distasonis*, a *Clostridium* sp. and an EOS fusiform bacterium. The National Cancer Institute decided to standardize the microflora and make it more representative of the natural microbiota, leading to the Altered Schaedler Flora (ASF) consisting of eight microbes **(Table 1.2)** (Orcutt *et al*, 1987).

One of the biggest problems with gnotobiotic mice with the ASF is monitoring the presence of the microbes and ensuring that there are no other microbes present. The current methods for this are very limited. The most prevalent methods are biochemical and microbiological in nature. Since many of the bacteria are slow growing, this process is tedious (Dewhirst *et al*, 1999). While most germ-free mice are assumed to be pathogen free, there is no steadfast way to ensure that the pathogenesis of the disease is solely due to the agent being studied.

An interesting aspect of infection is to study the interaction of pathogens with endogenous microflora. There have been reports of differences in disease response in animal models in the presence of microflora as opposed to germ-free animals (Gordon *et al*, 1971). For example, the addition of *Lactobacillus* spp. to the microflora protected

mutant and inbred mice from inflammatory bowel disease caused by the pathogen *H. hepaticus* (Madsen *et al*, 1999).

Table 1.2: Bacteria in the Altered Schaedler Flora

	Taxon(identity)	**Genbank accession no.**
ASF 356	Fusiform EOS bacteria (*Clostridium* sp.)	AF157052
ASF 360	*Lactobacillus acidophilus*	AF157050
ASF 361	*Lactobacillus salivarius*	AF157049
ASF 457	Spiral shaped org. (*Flexistipes* phylum)	AF157055
ASF 492	Fusiform EOS bacteria (*Eubacterium plexicaudatum*)	AF157054
ASF 500	Fusiform EOS bacteria (*Clostridium* sp.)	AF157051
ASF 502	Fusiform EOS bacteria (*Clostridium* sp.)	AF157053
ASF 519	*Bacteroides distasonis*	AF157056

The study of an infection of the gastrointestinal tract in small animals like mice leads to one methodological problem however: procedures like endoscopy are not possible and hence tissue sections would have to be taken only after sacrificing the animal. This would mean that long term studies would not be possible without the use of large numbers of animals. This problem can be overcome by using fecal matter as a surrogate indicator for the conditions existing in the gastrointestinal tract. Use of fecal pellets would lead to problems in culture related methods due to the death of many of the extremely oxygen sensitive bacteria that are known to colonize the GI tract. It would, however, be a valid assumption in nucleic acid based detection methods. In the studies I

conducted, I have developed molecular methods for the study of ASF species in murine fecal pellets. Such methods would allow the assessment of microbial population changes, in a reproducible and sensitive manner, without sacrificing the mice. The specificity of molecular methods, their accuracy and the time taken as compared to conventional culture-based methods makes them very suitable for this purpose.

Chapter 2

Design and testing of primers for *H. pylori* and the *Helicobacter genus*

Introduction

Helicobacter pylori is a gram negative microaerophilic bacterium that has been implicated in causing gastritis, peptic ulcers and is one of the factors that increases the risk of gastric cancers (Blaser, 1990). It infects about half the world's population, making it the most common pathogen found (Taylor *et al*, 1995). While the transmission of the organism from one host to another has been extensively studied and both oral-oral and fecal-oral transmissions have been reported, there is no conclusive proof for either of the modes. Environmental reservoirs for the pathogen other than human beings are not known, although it has been found in water (Hulten *et al*, 1996). This may, however, have been due to fecal contamination. The study of these and other questions is very dependent on the sensitivity and accuracy of the different detection methods used. The prevalent methods like culturing, breath tests and tissue urease tests face the problem of either taking too long or not being sensitive to low pathogen loads. Also, the organism often enters a coccoid form that is viable but non-culturable. This makes detection by culture tests difficult.

These factors have led to an increased investigation of PCR based methods for the detection of *H. pylori* in clinical and environmental samples. PCR based methods have the advantage of being extremely sensitive and robust. Another added advantage with PCR is that the method can be used to reliably quantify pathogen load in samples in a

very short period of time, provided the primers used are well designed. PCR based methods have been used to detect *H. pylori* in a wide range of samples ranging from dental plaque (Riggio *et al*, 1999), gall bladder and bile stones (Monti *et al*, 1999) to stool samples (Enroth *et al*, 1995) and house flies (Grubel *et al*, 1998). Several groups have developed primers for *H. pylori*. The targets that have been used include the 16S rRNA gene, which we have used in our work, and the urease genes. Urease genes like *ureC* are relatively unsuitable targets due to the fact that the database of organisms that express urease is not very large and there may be convergent sequences in closely related organisms for the urease genes, leading to false positives. The 16S rRNA gene is commonly used to detect organisms because of its ubiquity and the large database of 16S sequences that exists. It is present in two copies in the *H. pylori* genome and has been shown to have a sequence variation of only 0.2 to 0.5% between different strains. This makes it an attractive target for molecular analysis.

The use of the 16S gene for detection of *H. pylori* was pioneered by Morotomi *et al* (1989), who developed a 16S rRNA gene based oligonucleotide probe for the detection of *H. pylori*, then known as *Campylobacter pylori*. Ho *et al* (1991) were one of the first groups to utilize a PCR based strategy for the detection of *H. pylori*. They designed primers that targeted the 16S gene to produce a 109bp product. This pair of primers has been widely used to detect *H. pylori* in various clinical specimens like gastric biopsy samples, gastric juice, dental plaque, saliva and stool by various groups. However, Chong *et al* reported that the primer set Hp1-Hp2 developed by Ho *et al* gave many false positives when used with tissue samples due to amplification of a product of a similar size from the human genome. A nested PCR approach was advocated for more

accurate results with this primer pair (Chong *et al*, 1996). A BLAST search on the primers indicated that the primer Hp2 has a 3bp mismatch with some strains at the 3' end, which would probably reduce its efficiency (unpublished results). The primer Hp3 designed by Ho *et al* is from a region in the 16S gene conserved between many of the helicobacters. This primer also amplifies human DNA sequences. Smith *et al* (1996) also designed primers for the 16S rRNA gene that amplify a 495bp product. One of their primers, however, displayed a 3bp variation between *H. pylori* strains.

Other helicobacters like *H. hepaticus* are known pathogens of animals and the number of helicobacters known is growing. Since most helicobacters are pathogens, a method to detect all helicobacters would be very beneficial. Such a method would enable studies of co-infection of animals with different helicobacters and if proven robust enough, would make identification of new helicobacters easier.

The aim of my work is to design specific and sensitive primers for *H. pylori* and the *Helicobacter* genus based on a sequence alignment of currently available *Helicobacter* 16S gene sequences. The specificity and sensitivity of the primer pairs was tested experimentally against an array of helicobacters. Since the use of these primers will be in murine samples in our research, we also test the sensitivity of the primers in a background of DNA extracted from murine fecal pellets.

Materials and methods

Alignment of sequences

Initially, the *Helicobacter pylori* 16S rRNA gene sequence was manually aligned with the *E. coli* 16S rRNA gene based on the *E. coli* gene secondary structure (Woese *et*

al). An *H. pylori* 16S rRNA sequence (Genbank accession no. M88157) was chosen for alignment with the secondary structure, as it was the most complete 16S sequence that could be found in Genbank, with the fewest ambiguous nucleotide positions. This provided a robust analysis of the sequence for any possible sequencing errors and laid the basis for all further alignments. Subsequently, a universal alignment of the 16S rRNA gene from *E.coli* K-12 obtained from the Ribosomal Database Project was aligned with 16S rRNA gene of *H. pylori* (three different strains), *H. nemestrinae*, *H. felis*, *H. canis*, *H. cinaedi*, *H cholecystus*, *H. salomonis*, *H. "Mainz"*, *H. bilis*, *H. pullorum*, *H. trogontum*, *H. felis*, *H. heilmanii*, *H. muridarum*, *H. rodentium*, *H. mustelae*, *H. suncus*, *H. pametensis*, *H. hepaticus*, *Flexispira rappini* and *Wolinella succinogenes* using the alignment editor Seqlab (Genetics Computing group, Madison, WI).

Primer design

H. pylori specific primers

Different regions with marked sequence variation between *H. pylori* and the rest of the sequences were chosen for primer design. Short sequence stretches from within these regions were used as inputs for BLAST. Sequences that showed exact sequence match with the *H. pylori* 16S genes and with a maximum of one or two other species were chosen for further analysis. The sequence analysis program MacVector (Oxford Molecular, Oxford, UK) was used to choose the primer sequence with a GC content in the 40-50% range. The program also provided a detailed analysis of the possibility of primer self-annealing and primer dimer formation. Due to the low GC content in the regions containing the primer sequence for 976f, 1017r and 1127r, these primers had to

be 29bp, 26bp and 30bp long respectively, so as to have ensure optimal melting temperatures.

Helicobacter genus specific primers

Genus specific primers were designed in a manner similar to that described above. Regions showing sequence identity across all the helicobacters and other species that are closely related to the helicobacters, based on sequence data, were chosen for primer design. Short sequence stretches from the selected regions were analyzed using BLAST. All the primers were ordered from Integrated DNA Technologies, Inc.(Coralville, IA).

PCR analysis of primer pairs

All polymerase chain reactions were run on a RoboCycler™ 96 thermocycler (Stratagene, La Jolla CA). PCRs were performed with 20mM Tris-HCl (pH 8.4), 50mM KCl, 0.2mM each of dATP, dGTP, dCTP and dTTP, 2mM $MgCl_2$ and 0.5U Taq Polymerase (Life Technologies, Rockville, MD). All reactions had a final volume of 20µl. Primer concentrations were kept constant at 100nM for the whole series of experiments. The reactions typically involved variable numbers of cycles (30-35) of 1 minute at 94°C (denaturation) followed by 30 seconds at the annealing temperature and 2 minutes of extension at 72°C. Optimal annealing temperatures were identified for each primer pair by using the temperature gradient feature of the thermocycler.

The results of PCR were viewed on a 1% ultrapure Agarose gel (Life Technologies, Rockville, MD). The buffer used for gel electrophoresis was 0.5X Tris-

borate-EDTA (TBE). Gels were photographed using the EagleEye™ gel documentation system (Stratagene, La Jolla, CA).

The amount of template used was often difficult to quantify due to the limited quantities of genomic DNA template available. The concentration of the *H. pylori* DNA stock was measured to be 19ng/μl by absorbance measurements at 260nm. About 2μl of stock *H. pylori* genomic DNA was used for each reaction resulting in a final concentration of approximately 40ng per 20μl reaction. The purity of DNA used was checked on an agarose gel. For sensitivity assays using the *H. pylori* specific primers, the stock was diluted in a series of ten-fold dilutions to a 10^{-9} dilution . The sensitivity assay was performed with *H. pylori* genomic DNA templates ranging from 38 ng/reaction to 3.8×10^{-8} ng/reaction. Calculations for this part are included in the appendix B.

Results

H. pylori specific primers

Design of the primers

Four regions were identified in the alignment with uniformly high sequence difference between *H. pylori* and the other helicobacters. These regions were used for designing 4 species specific primers.

787f: The primer sequence was found in all the available sequences for the *H. pylori* 16S gene. The closest match to the sequence of the primer is the corresponding region of the 16S rRNA sequence in *H. nemestrinae*, which is identical (**Appendix A, Figure A.1**). The next closest match to the primer sequence is that of *H. suncus*, which has three differing bases and two deletions. A BLAST search on the primer sequence matched the *H. pylori* and *H. nemestrinae* 16S gene.

976f: The primer sequence is specific for all *H. pylori* strains and strain 85D08. (**Appendix A, Figure A.2**) There is a difference of two nucleotide positions between *H. pylori* strain 43504 in the alignment and the sequence from *H. pylori* strain 85D08. A secondary structure model revealed that the differing bases are complementary and are present in a stem loop structure. Thus, it is unlikely that the differing bases are a sequence artifact. There are one base pair differences between the primer sequence and the corresponding regions in the alignment of *H. felis*. A BLAST search gave a perfect sequence match for *H. acinonyx*.

1017r: There were two degeneracies between the main *H. pylori*16S sequence in the alignment (Hp43504) and another *H. pylori* sequence from Genbank (strain 85D08) at positions 2 and 9. From the alignment of the *H. pylori*16S rRNA gene with the secondary structure of the *E. coli* 16S rRNA gene, these nucleotide positions are complementary in a stem loop structure. Since the copy number of the 16S rRNA gene in *H. pylori* is 2, it is possible that there may be slight variations in sequence between the two operons, as has been reported for *E. coli.* An alignment of the two operons from the strain 26995 shows a deletions and base mismatches between the two copies of the gene. This degeneracy would indicate that the primer may not amplify all the *H. pylori* strains with the same efficiency. In addition, *H. canis* is a perfect match for the primer sequence and *H. felis* has only a one nucleotide difference, at position 15 in both the strains included in the alignment. *H. rodentium* also has one nucleotide different at position 13 in the alignment. The closely related *Helicobacter nemestrinae* has 5 nucleotides different in the primer region. A BLAST search for the primer sequence yielded perfect matches for *H. pylori* and *H. canis* only (**Appendix A, Figure A.3**).

1127r: This region from which this primer was selected had a single nucleotide difference between the main *H. pylori* strain in the alignment and *H. pylori* strain 85D08. *H. nemestrinae* has one nucleotide different and a deletion within the primer region. Based on sequence similarity, this primer appears to be much better than 1017r with widespread variation over all the species included in the alignment (**Appendix A, Figure A.4**). There are at least three nucleotides different for *H. felis* and *H. canis* has 5 positions

different coupled with a deletion. A BLAST search on the primer sequence yielded exact sequence matches for *H. pylori* only.

Testing of primer pairs:

Three different combinations of the above primers (787f-1017r, 976f-1127r and 787f-1127r) were evaluated empirically with nine different helicobacters and two *H. pylori* strains. The strategy employed was to use a combination of two primers where each had a perfect match for an organism other than *H. pylori* (787f – *H. nemestrinae*, 1017r: *H. canis*). This approach would be specific even if one of the primers is a perfect match for a Helicobacter other than *H. pylori*. The results are summarized in **Table 2.1**. The primer pair 976f-1017r was not tested as the length of the PCR fragment would be about 50bp and the fragment would be of little use in applications due to its size. In initial testing, 976f-1127r gave a PCR product of size ~150bp with *H. pylori* and a product of the right size with *H. bilis* also. Hence, further testing of this primer pair was not carried out. This may, however, been due to impure *H. bilis* template.

The primer pair 787f-1017r gave a band (size ~230bp) with *H. pylori* and *H. mustelae* and was, thus, not further pursued (data not shown). The primer pair 787f-1127r gave a band only with *H. pylori*. The size was ~330bp as expected. (**Figure 2.1**) The lack of a band with *H. canis* for 787-1017r is of significant importance because it is an exact match for the reverse primer 1017r. This indicates a success of our strategy of using primers which both had specificity for *H. pylori* and a *Helicobacter* different for the

forward and reverse primer.The optimal annealing temperature for both the primer pairs 787f-1017r and 787f-1127r was 56°C.

To test the sensitivity of the two primer pairs, a dilution series was set up in a constant background of about 100ng of DNA extracted from mice fecal pellets (as measured by absorbance at 260nm). Concentration of DNA in the *H. pylori* G27 genomic DNA (obtained from D. Schauer) was 19ng/ul, as measured by absorbance at 260nm. This was calculated to be 4 x 10^7 copies of the 16S gene, based on the assumption that the genome was approximately 1.67 x 10^6bp long. Sensitivity based on a single PCR followed by detection on a 1% agarose gel was 20 copies (10^{-6} dilution) for 787f-1127r (**Figure 2.2**) and 200 copies (10^{-5} dilution) for 787f-1017r. (Data not shown). The detailed calculation is included in **Appendix B**. Since all detection *of H. pylori* is in animal samples, in a background of non-specific DNA, the primer pairs are extremely sensitive for this purpose. The results are also limited by the detection limit of the agarose gel and the actual limit of detection may be even lower with a more sensitive detection method, like detection using fluorescently tagged primers. A second PCR step with the PCR products of the dilutions 10^{-7} to 10^{-9} gave a band of the correct size for detection of a single copy of the target (10^{-7} dilution). (data not shown) The specificity and sensitivity of the primer pair 787f-1127r makes it a good pair for applications like quantitative PCR.

TABLE 2.1. Evaluation of *Helicobcater pylori* primers (empirical)

Species	**787f-1017r**	**976f-1127r**	**787f-1127r**
H. pylori	++	+	++
H. bilis	-	+	-
H. rodentum	-	-	-
H. fenneliae	-	-	-
H. cinnaedi	-	-	-
H. cholecystus	-	-	-
H. hepaticus	-	-	-
H. mustelae	++	nt	-
H. canis	-	nt	-
H. pullorum	-	-	-
ASF502	-	nt[a]	nt
ASF519	-	nt	nt

[a] nt = not tested

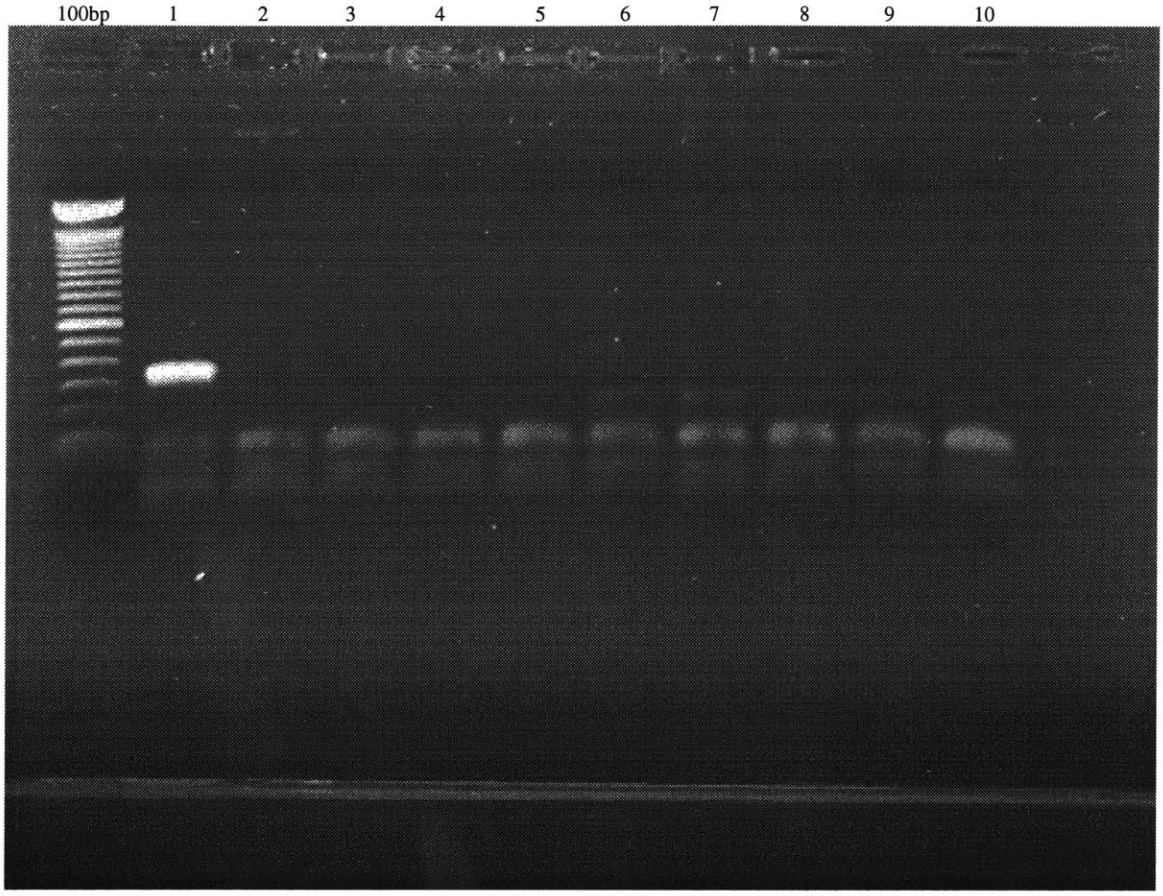

Figure 2.1: The specificity of primer pair 787f-1127r was tested using various *Helicobacter* species. The lanes marked 1 to 10 are : *H. pylori, H. bilis, H. rodentium, H. pullorum, H. hepaticus, H. cholecystus, H. cinnaedi, H. fenneliae,* Mice fecal pellet DNA and the negative control (no DNA). The bands present in all the lanes are primer dimers.

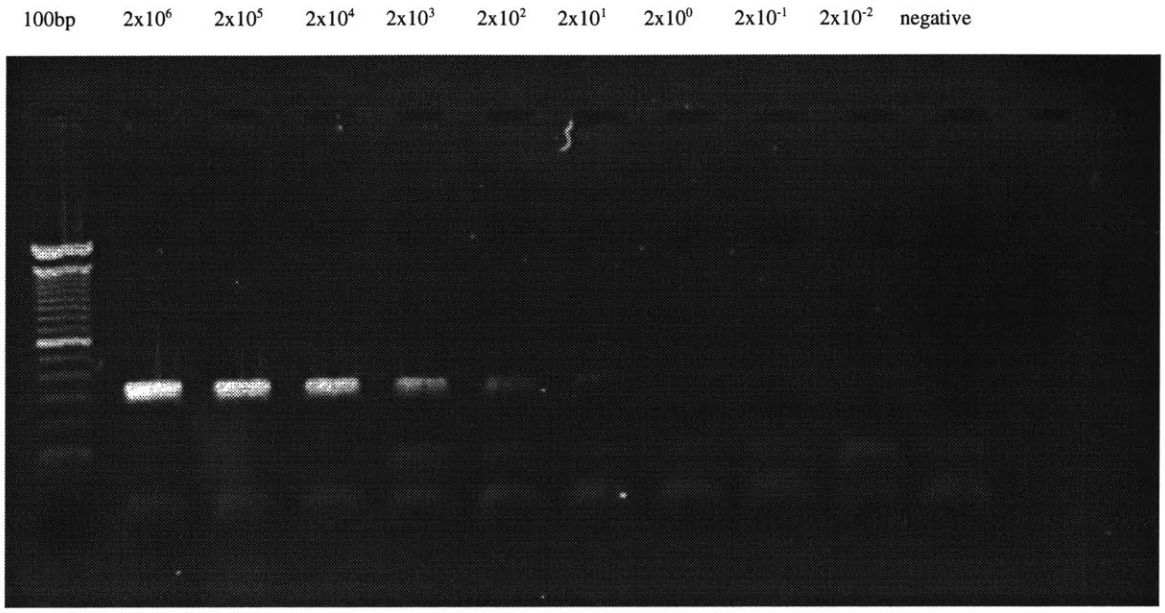

Figure 2.2: Sensitivity of H. pylori specific primer pair 787f-1127r in a background of mice fecal pellet DNA. The background is kept constant while *H. pylori* DNA is in serial dilution. The numbers above the lane indicate the number of copies of template, as calculated in Appendix B.

***Helicobacter* genus specific primers**

Design of the primers

Three regions with a high degree of sequence identity for all helicobacters were used to design *Helicobacter* genus specific primers.

212f: The region from which this primer was designed shows a high level of sequence similarity for most of the helicobacters in the alignment, except for *H. felis*, *H. salomonis* (3 nucleotides different in each), *H. pullorum, H. trogontum, H. felis, H. heilmanii* (2 nucleotides different in each) and *H. "Mainz"* and *H. mustelae* (1 nucleotide different). **(Appendix A, Figure A.5)** The BLAST results for the primer sequence include all the helicobacters and the closely related species, *Flexispira rappini* and *Wolinella succinogenes.*

289r: The region for the design of this primer has a perfect sequence match for all the helicobacters included in the alignment. **(Appendix A, Figure A.6)** This makes this a very good choice for a genus specific probe, other than use as a primer. The BLAST results for the primer sequence yield perfect hits for helicobacters and an organism in the *Treponema* and *Corynebacterium* genus.

1026r: The sequences aligned in this region show perfect sequence identity for all the species **(Appendix A, Figure A.7)**. BLAST results for the primer sequence show a perfect sequence identity with a few bacteria isolated from extreme environments other than the predominant *Helicobacter* hits. None of the other bacteria are from sources other than extreme environments.

Testing of primer pairs :

The two possible primer pairs, 212f-289r (~100 bp fragment) and 212f-1026r (~820bp) were tested with ten *Helicobacter* species and two unrelated organisms (**Table 2.2**). All the helicobacters tested amplify well with both primer pairs. The primer pairs 212f-289r and 212f-1026r had an optimal annealing temperature in the range of 56-58°C. However, non-specific PCR products of several different sizes were produced when DNA from *Bacteroides* sp. (ASF 519) and *Clostridium* sp. (ASF 502) was used as the template. This could be due to the length of the primers, which may reduce their specificity in annealing. Thus, the primers are being redesigned to shorten them.

TABLE 2.2. Evaluation of *Helicobacter* genus specific primers (empirical)

Species	**212f-289r**	**212f-1026r**
H. pylori	++	++
H. bilis	++	++
H. rodentum	++	++
H. fenneliae	++	++
H. cinnaedi	++	++
H. cholecystus	++	++
H. hepaticus	++	++
H. pullorum	++	++
ASF502	Non-sp[a]	Non-sp
ASF519	Non-sp	Non-sp

[a] Non-specific bands were seen

Discussion

PCR is a powerful tool for the detection and quantification of microorganisms. There are several parameters that govern the utility of PCR for the purpose of diagnostics. The primer sequence, melting temperature, GC content and secondary structure formation are a few of the factors that can affect the accuracy of the method. One of the crucial factors that governs the utility of the method is the quality of the primers. Primers are designed by the comparison of sequences of a target gene with the database of all sequences and choosing short nucleotide sequences that have unique presence in the target of interest. All of the above factors are taken into consideration for the design of primers. The detection of *H. pylori* by PCR methods has been commonly reported by many groups (Ho *et al*, 1992, Smith *et al*, 1996, Enroth *et al*, 1995, Chong *et al*, 1996) and has been shown to be a sensitive and rapid method of detection of *H. pylori*.

The design of primers in this study was based on a rigorous procedure involving the additional step of comparison of secondary structure based models of the 16S rRNA of *H. pylori* and the model for *E. coli* in addition to sequence alignment with most of the helicobacters known, in addition to the sequence comparison approach mentioned above. However, given the increasing number of helicobacters being discovered, it is important to continually update the alignments to ensure the accuracy of the primers. Although the empirical testing of the primers was very extensive, the inclusion of more strainswill be needed for accuracy of the primers. The differences in a few nucleotides between different *H. pylori* strains as seen in the alignments indicate that a more extensive testing of the primers with different *H. pylori* strains would support the utility of the primers for all strains. The primer pairs tested hit all strains of *H. pylori* in the alignment except for

the one with nucleotide differences. An alternate strategy that can be used is the introduction of degeneracies in the primers designed from regions that exhibit sequence differences for different strains of *H. pylori*.

Of the three primer pairs 787f-1017r, 787f-1127r and 976f-1127r, the primer pair 787f-1127r was found to have the best specificity and sensitivity. The other primer pairs amplified helicobacters other than *H. pylori* and were hence not further analyzed. However, they amplify the correct sized fragment in *H. pylori*.

The detection limit of 20 *H. pylori* bacteria with the primer pair 787f-1127r in a background of mice fecal pellet DNA is a very good sensitivity. Ho *et al* reported a detection limit of 0.1pg of *H. pylori* genomic DNA with 40 cycles, while the primer pair 787f-1127r could detect 0.03pg of genomic DNA, in a background of mice fecal pellet DNA, in 35 cycles. However, this data has to be validated with mixtures of *H. pylori* cells and fecal pellets. Furthermore, the calculation of sensitivity (Appendix B) is based on the assumption that the absorbance data for DNA concentration was an accurate measure. The use of actual dilutions of known numbers of whole *H. pylori* cells would provide a better estimate of the sensitivity. The sensitivity and specificity of the primer pair would make it extremely appropriate for applications such as accurate detection and quantitation of *H. pylori* in lab and clinical samples.

The genus specific primer pairs 212f-289r and 212f-1026r amplified the correct sized fragment from all the helicobacters tested. However, both the primer pairs produced multiple bands with bacterial DNA extracted from fecal pellets free of helicobacters and with bacterial DNA from *Clostridium* sp. ASF502 and *Bacteroides distasonis* (ASF519). One of the reasons for the non-specific bands could be the fact that the forward primer

212f was 30 nucleotides long. This would increase the probability of a reduction in the specificity of annealing. The primer is currently being redesigned and will also be tested with a larger range of helicobacters to ensure universality of amplification within the *Helicobacter* genus. The fact that these primers amplify the correct sized fragment in all the helicobacters tested indicates their utility. With an increase in specificity that can potentially be achieved by modifying the primers, they should prove a valuable tool in the detection and study of helicobacters.

Chapter 3

Methods for diversity analysis of ASF species in the mouse model

Introduction

The gut is a region of enormous microbial loading and diversity. A recent study (Suau, 1999) indicated that there were 85 species of bacteria present in the human gut of which only a fourth were known species. The bacterial loading in the gut can often be as high as 10^{11} bacteria/gram of feces. Thus, the gut ecosystem is an extremely complex one, which plays a very important role in normal digestion and health. Some of these microbes help in providing nutrients that the hosts are unable to make themselves, such as vitamin K. Others may occupy niches that help protect the host from pathogens. Several factors, including antibiotics and pathogens, can affect the intestinal microflora. Given the recently discovered role of some of the members of the microflora in reducing the severity of disease in laboratory animals, these interactions are an interesting and important field for research.

The study of the microflora is hindered by its extreme complexity. Culture methods would capture only a fraction of the diversity, as many of the bacteria are extremely oxygen sensitive (EOS) bacteria. Molecular methods for the study of such a complex community would be unable to resolve the different species and would be time-consuming. Thus, a defined microbiota that is representative of the gut microflora would be extremely useful as a system for studying the ecology of the gut system.

One such system is the mouse model inoculated with the Altered Schaedler flora. The original Schaedler flora developed in the the mid-1960s was a mixture of 8 microbes: *E. coli* var. *mutabilis*, *Streptococcus faecalis*, *Lactobacillus acidophilus*, *Lactobacillus*

salivarus, group N *Streptococcus*, *Bacteroides distasonis*, a *Clostridium* sp. and an extremely oxygen sensitive (EOS) fusiform bacterium. This mixture was skewed from the normal distribution in the gut, which has a lot of EOS bacteria. Thus, in 1978, this was altered and the new flora was called the Altered Schaedler flora (ASF). A recent study characterized the ASF species on the basis of their 16S rRNA sequences. (Dewhirst *et al*, 1999)

The monitoring of the ASF flora in germ-free mice is still a very difficult proposition. It is not possible to show that only these bacteria colonize the mice by conventional culture and biochemical methods alone. Further, culture methods are very tedious for many of the EOS bacteria. This makes molecular methods for the study of this system extremely useful.

The problems faced in the study of gut microflora are similar to those faced in the study of the ecology of complex environments, e.g. the microbial population in an activated sludge reactor. Thus, methods used in molecular ecology studies would be useful tools in the gut ecosystem too. Two different approaches have been used in this study, terminal restriction fragment polymorphism (t-RFLP) and constant denaturant capillary electrophoresis (CDCE). Both approaches require the amplification of DNA extracted from fecal pellets by universal 16S primers, with one of the primers being fluorescently labeled. In t-RFLP, the resultant mixture of PCR products from all the ASF species is then digested with a set of specially selected restriction enzymes to yield terminal fragments of different lengths at the fluorescent primer end for each of the ASF species. These fragments of different length are then separated according to their length by capillary electrophoresis on an automated sequencer. This method was originally

developed for the analysis of mycobacteria by Avannis-Aghajani *et al* (1996) and has subsequently been used by many researchers for analyzing microbial community structure in natural habitats (Liu *et al*, 1998, Marsh *et al*, 1998). In CDCE, the principle of separation is the difference in melting temperatures between different DNA sequences. PCR products are analyzed by capillary electrophoresis at a temperature at which the amplicon from each species is in a different stage of melting and hence runs with different mobility in the gel. Since the melting temperature is different for sequences with even a single base pair variation, this is an extremely sensitive method (Khrapko *et al*, 1994).

The aim of this research is to develop molecular methods that allow us to study the ASF microflora as a group without culturing them, by the use of the two methods described above.

Materials and methods

t-RFLP

Choice of restriction enzymes for analysis

The utility of this method for analyzing the ASF species diversity is dependent on the choice of restriction enzymes that yield unique lengths for each of the species after digestion. The 16S rRNA gene sequences for all the ASF species were obtained from Genbank. Each of the sequences was analyzed for the 5' terminal fragment size upon *in silico* digestion by all the restriction enzymes on RestrictionMapper, a graphical user interface tool for restriction mapping. Based on this analysis, it was determined that one enzyme would not be able to resolve all 8 species. A combination of two enzymes, *HhaI* and *NciI*, resolved the 8 species into fragments differing by at least 6bp.

Fecal pellets and ASF species' DNA

Fecal pellets from ASF mice were obtained from Dr. D. Schauer (MIT). Crude DNA extracts from individual ASF species were kindly contributed by Dr. B. Paster (Forsyth Institute, Boston) and R. Peña (MIT).

Extraction of DNA from fecal pellets

DNA was extracted from fecal pellets by bead-beating (personal communication with Dr.D. Stahl). Fecal pellets were resuspended in phosphate buffer saline (PBS, pH 7.4). This suspension was added to a tube with zirconium beads in it. 500µl of equilibrated phenol and SDS were added to the mixture and the tube was shaken on the bead beater at high speed for 2 minutes. This was followed by an incubation at 60°C for 2 minutes. The tubes were then shaken on the bead beater at maximum speed for 1 minute. Subsequently, a centrifugation at 12000 RPM for 10 minutes was performed and the supernatant obtained was mixed with an equal volume of phenol:chloroform:isopropanol. A cenrifugation step for 5 minutes at 12000 RPM followed. The aqueous phase was separated and an equal volume of chloroform was added to it, followed by another centrifugation at 12000 RPM for 5 minutes. The supernatant was separated and the DNA was precipitated with 100% cold ethanol at 0°C for 20 minutes in the presence of sodium acetate. Then, a centrifugation at 12000 RPM for 15 minutes was done. The supernatant was discarded and the DNA pellet was dried in a vacuum in a SpeedVac. The pellet was then resuspended in 100µl water and stored for use at -20°C.

Primers

The primers used in the analysis were the universal 16S primers 27F and 1492R, which amplified an approximately 1450bp long fragment of the 16S gene (**Table 3.1**). The forward primer was modified with a 5' HEX (phosphoramidite dye) label.

Table 3.1: Primers used in analyses

Primer	Method	Sequence[a]
27f (universal, 16S)	t-RFLP	5' AGAGTTTGATCMTGGCTCAG 3'
1392f (universal, 16S)	CDCE	5' GYACACACCGCCCGT 3'
1492r (universal, 16S)	CDCE[b], t-RFLP	5' TACGGYTACCTTGTTACGACTT 3'

[a] The notation used for degenerate nucleotide positions is as follows : M = C: A, Y= C:T,
[b] For CDCE analysis, the reverse primer was modified to have a GC clamp (5' GCGGGCGGCGCGGGGCGCGGGCAGGGCGGCGGGGGCGGGC 3') and a 5' FITC label.

PCR

PCR reactions were performed on a RoboCycler™ 96 thermocycler (Stratagene, La Jolla CA) with 20mM Tris-HCl (pH 8.4), 50mM KCl, 0.2mM each of dATP, dGTP, dCTP and dTTP, 2mM $MgCl_2$ and 0.5U Taq Polymerase (Life Technologies, Rockville, MD). All reactions had a final volume of 20µl. Primer concentrations were kept constant at 100nM for the whole series of experiments. The reactions involved 35 cycles of 1 minute incubation at 94°C (denaturation) followed by 30 seconds at 50°C and 2 minutes of extension at 72°C.

Restriction digestion and sample preparation

The restriction enzymes *HhaI* and *NciI* and their buffer (NEBuffer 4) were purchased from New England Biolabs, MA. 15µl of PCR product was incubated with 6 units of each enzyme, 1x concentration of NEBuffer 4 (50mM potassium acetate, 20mM tris-acetate, 10mM magnesium acetate, 1mM dithiothreitol) and 100µg/ml BSA at 37°C overnight, followed by a 20 minute incubation at 65°C. Initially, incubations were for 3 hours, but this led to partial digestion products and the incubation time was increased to overnight digestion. Digested products were then purified with Qiaquick PCR purification kit (Qiagen, CA) to remove unincorporated primers, nucleotides, nucleotide triphosphates and primer dimers.

Analysis of purified samples

Purified samples were then analyzed on the ABI310 Genetic Analyzer in the GeneScan mode, which essentially separates end-labeled DNA fragments based on size differences, with a 1-2bp resolution, using laser induced fluorescence in the HEX end label. Samples for GeneScan were prepared by adding 1µl of the purified product from restriction digestion to 12µl of high-purity formamide (Perkin-Elmer, CA) and 0.5µl of GS500-TAMRA size standard, which has size standards in the range of 35-500 nucleotides. Samples were electrophoresed in POP-4 polymer gel at 60°C for 30 minutes at 10kV. The resultant spectral data was analyzed using the GeneScan software to obtain electrophoretograms for the runs.

CDCE analysis

Primers

The primers used in sample preparation for CDCE analysis were universal 16S primers, 1392F and 1492R. The reverse primer had a 40bp GC clamp attached to it and was labeled with FITC. (**Table 3.1**) (Synthetic Genetics, CA)

PCR

PCR reactions (20µl reaction volume) were performed on a RoboCycler™ 96 thermocycler (Stratagene, La Jolla CA) with 20mM Tris-HCl (pH 8.4), 50mM KCl, 0.2mM each of dATP, dGTP, dCTP and dTTP, 2mM $MgCl_2$ and 0.5U Pfu Polymerase. Pfu was used as it has a 3'-5' exonuclease activity and a proofreading mechanism that leads to fewer errors. This is of critical importance in our protocol, as the method is so sensitive that it can be skewed by single base pair changes. Pure species DNA for each of the ASFs was used as the template. The PCR reactions involved 35 cycles of 1 minute incubation at 94°C (denaturation) followed by 30 seconds at 50°C and 2 minutes of extension at 72°C. This was followed by an additional 10 minute incubation at 45°C.

CDCE analysis

0.3µl of the PCR product was diluted to 3µl and loaded into the capillary at 2mA for 30 seconds. The sample was run at different temperatures that were controlled by a water jacket around the capillary. The samples were run at 5kV and 9mA for approximately 20 minutes each. Data from the fluorescence excitation of the label on the

reverse primer by the laser was recorded by the machine and converted to electropherograms.

Results

The aim of this work is to be able to distinguish clearly the different species present in a fecal DNA sample from an ASF mouse. The results from the t-RFLP runs on the ABI 310 Sequence Analyzer showed distinct peaks for the species tested. The CDCE method yielded results that were ambiguous, but amenable to explanation.

T-RFLP analysis

The basic principle of this method is that sequence diversity can be captured by the length variation of the terminal fragments obtained by digestion of a mixture of amplified 16S genes with appropriate restriction enzymes. The chief aim of the work done here was to test the utility of the method for the purpose of diversity assessment of the ASF species.

The critical step in the use of t-RFLP analysis for the ASF species is the choice of restriction enzymes. Various restriction enzymes were used for computer based analysis of the terminal fragment lengths of the ASF species and it was observed that one enzyme would not be able to resolve all 8 species into different sizes. Hence, *in silico* digestion with different enzyme pairs was tried and the enzymes *HhaI* and *NciI* were found to resolve the 8 species with a minimum terminal fragment length difference of 6bp.

Three different sets of experiments were then performed to standardize and test the method - analysis of single species digests to validate the *in silico* digestions, analysis of artificial mixtures of the 16S genes of the ASF species to standardize the method and

analysis of actual fecal pellet DNA samples from ASF mice to study the diversity of the ASF species.

The first set of experiments involved the amplification of pure species DNA from each of the ASF species followed by restriction digestion with *HhaI* and *NciI*. The resultant products were then run on an ABI 310 Sequence Analyzer in the GeneScan mode.

The fragment sizes detected were in the range expected, as shown in **Table 3.2.** The electrophoretogram for *Clostridium* sp. ASF 502 showed an extra fragment that corresponded to a digestion product. Initial digestion for 3 hours produced incomplete digestion products and so the digestion was extended overnight for all subsequent experiemens. In one of the pure species samples (*E. plexicaudatum*, ASF492), an extra fragment of a size that did not correspond to a product of incomplete digestion of the 16S gene product was observed. This could be attributed to contamination of this sample with other bacterial DNA. An example of the electrophoretogram for pure ASF species experiment is shown in **Figure 3.1**. The lengths of the fragments obtained by GeneScan analysis did not match the expected lengths exactly and there was a discrepancy between 1 to 6bp. The reasons for this seem to be related to the base-calling algorithm used. There are five algorithms that can be used: second order regression, third order regression, cubic spline, local southern and global southern. Such problems with the choice of algorithms have been reported in literature (Osborn et al, 2000) and can be resolved by comparing results obtained by using different algorithms.

The second set of experiments was carried out by amplifying mixtures of the 16S PCR products of individual species. This analysis was carried out for mixtures of two,

four and six different species. The results show the expected fragment sizes within the margin of error discussed above. The electrophoretogram for the mixture of two species is shown in **Figure 3.2.** This result validates the use of t-RFLP for the purposes of assessing diversity of the ASF mice. The third set of experiments was conducted with DNA extracted from fecal pellets obtained from three sets of ASF mice. The results indicate the presence of only three (Offspring 06/99, Breeding) or four (Offspring 04/99) species at a level detectable over the background. The fragment lengths present corresponded to *Bacteroides* sp., *Lactobacillus* sp. ASF 361, Clostridium sp. ASF356 (all three samples) and *Lactobacillus* sp. ASF360 (present only in the Offspring-04/99 sample). The electrophoretogram for the Offspring-04/99 sample is shown in **Figure 3.3.** The results for the pure species and the fecal pellet samples are summarized in **Table 3.2.**

Table 3.2: Preliminary results of t-RFLP experiments

Sample	Terminal fragment length[1, 3]	
Name (identity)	Expected	Observed
Pure species		
ASF 457(*Flexistipes* sp.)	93	91
ASF 519(*Bacteroides* sp.)	99	97
ASF 492(*E. plexicaudatum*)	151	151, 212[2]
ASF 502 (*Clostridium* sp.)	193	194,
ASF 361(*Lactobacillus* sp.)	257	257
ASF 500 (*Clostridium* sp.)	389	389
ASF 356 (*Clostridium* sp.)	480	474
ASF 360 (*Lactobacillus* sp.)	597	~590
Mice fecal pellet samples		
Offspring 04/99	-NA-	97, 252, 455, 590
Offspring 06/99	-NA-	97, 255, 455
Breeding	-NA-	97, 255, 455

[1] The restriction enzymes used in this analysis are *HhaI* and *NciI* (New England Biolabs). The reaction conditions were as follows: 12U of enzyme in a 20µl reaction volume with 10µl of PCR product, NEBuffer4 and BSA incubated at 37°C for 3 hours followed by a 20 minute inactivation at 65°C.

[2] Unexpected fragment size may be due to contamination or reannealing of single strands after sample preparation for GeneScan.

[3] The sizing of larger fragments with the current settings on the GeneScan software is sometimes inaccurate and the size calling algorithm has to be optimized for this system.

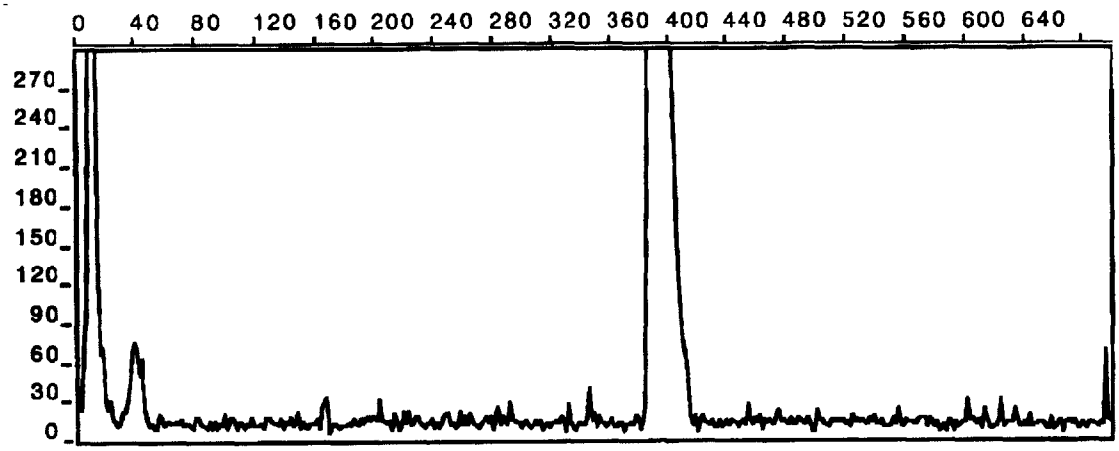

Figure 3.1: The electrophoretogram of a t-RFLP run for a single species, ASF500, which has an expected terminal fragment length of 389bp. The length by GeneScan analysis was found to correspond exactly with the expected length.

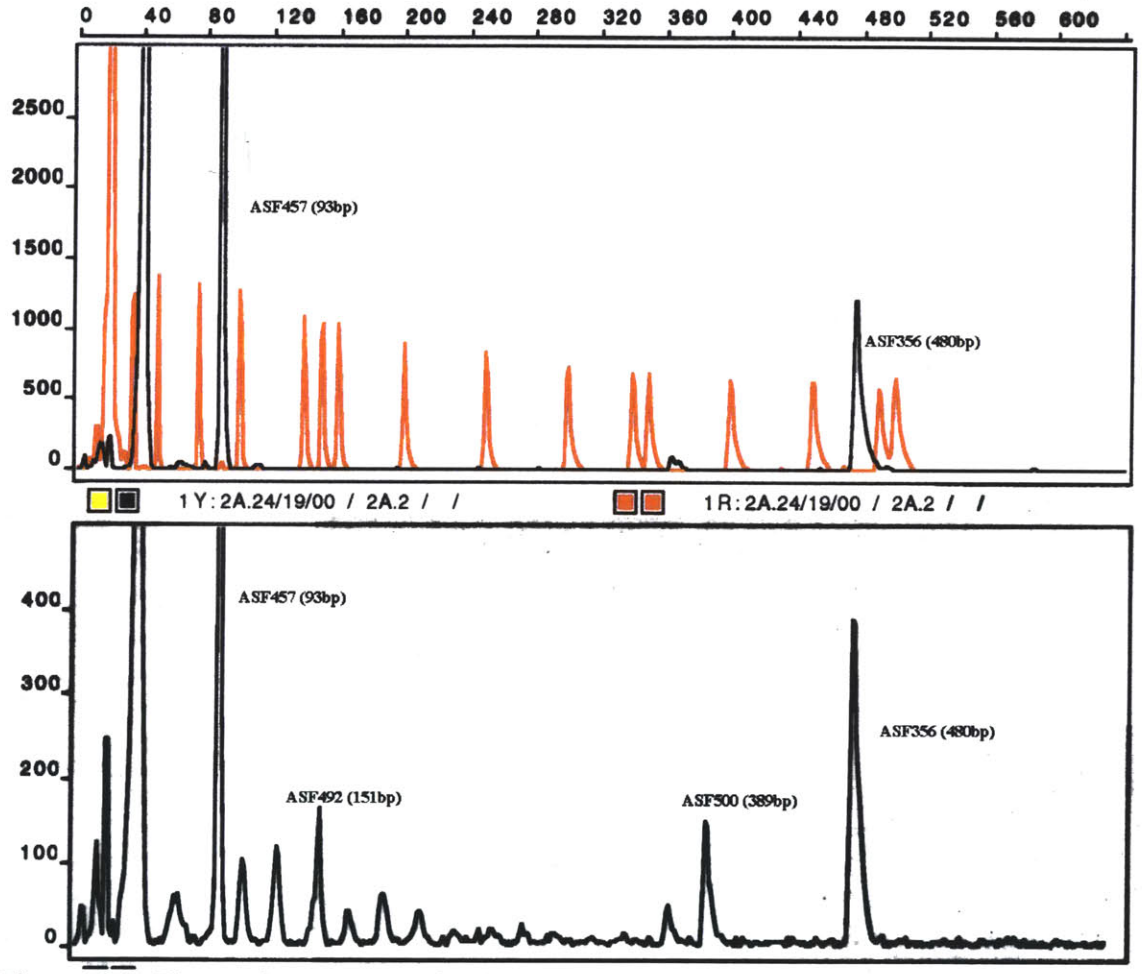

Figure 3.2: Electrophoretograms for the t-RFLP analysis of co-amplified mixtures of pure species templates. The top panel is a mixture of 2 species. The bottom panel is a mixture of 4 species. The size marker GS500 is in red in the top panel for comparison.

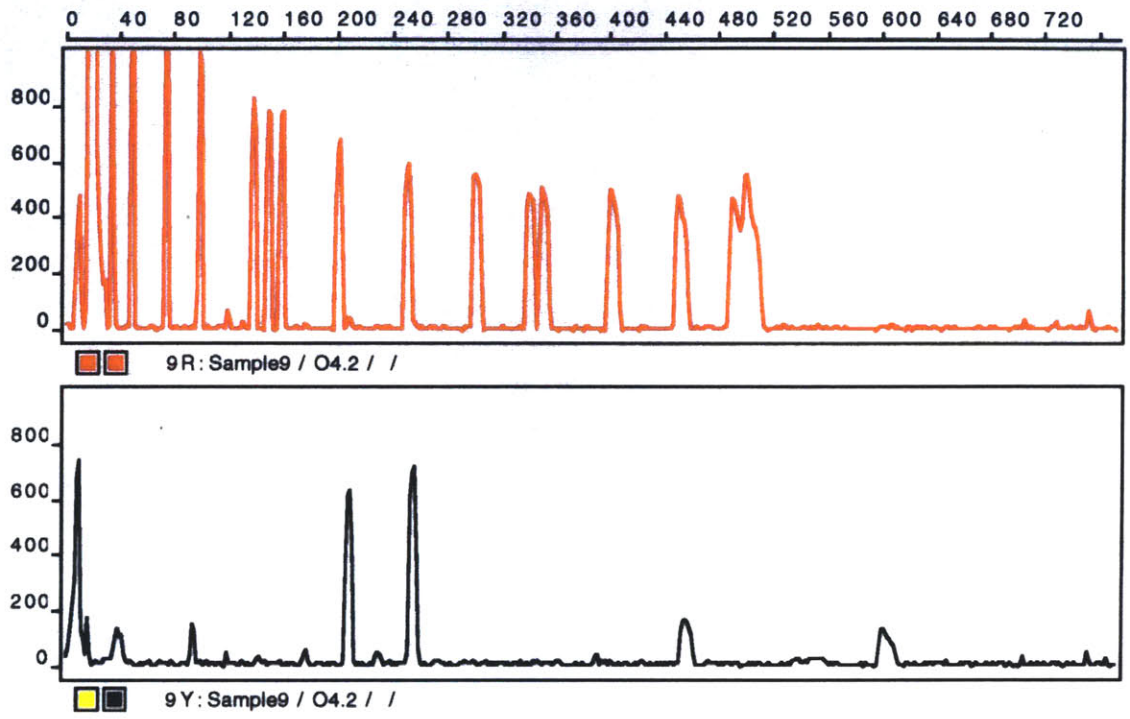

Figure 3.3: Electrophoretogram for the t-RFLP analysis of DNA extracted from fecal pellet sample Offspring 04/28. The upper panel shows the size marker DNA, GS500 and the lower panel shows the run result for the sample.

CDCE analysis

The utility of CDCE for studying the diversity of ASF species in mice fecal pellets was tested by trying to identify the peak for pure species samples. The melting profiles of the amplified fragments of the 16S rRNA gene for all the ASF species were plotted using MacMelt (Medprobe, Norway) and were found to be in the range of 74° to 80° C.

Three of the species, *Lactobacillus* sp. ASF361, *Clostridium* sp. ASF502 and *Bacteroides* sp. ASF 519 were chosen on the basis of their relatively low melting temperatures for standardizing the use of the method. The samples were analyzed individually in temperatures ranging from 74°C to 82°C. All the samples showed the separation of multiple adjacent peaks upon increasing the temperature. This was contrary to the single peak expected. This indicated that each pure species amplicon contained several different DNA sequences differing in a few nucleotides. The electropherogram for *Lactobacillus* sp. ASF361 is shown in **Figure 3.4**.

There are two possible explanations for observing multiple peaks for single species. One possibility is dependent on the fact that the primers used in the analysis were degenerate (**Table 3.1**). This leads to the formation of a mixture of several different PCR products, with varying amplification efficiency. The most abundant product would be the one with the primer sequences matching the target sequence, while other products would be formed with lesser efficiency. This explanation is borne out by the fact that the peaks in **Figure 3.4** are of varying heights. Another possibility is based upon the polymorphisms observed in different copies of the 16S SSU rRNA gene in bacteria.

Several copies of the gene differing in a few nucleotide positions in the species tested would lead to separation of the PCR products into separate peaks for a single species.

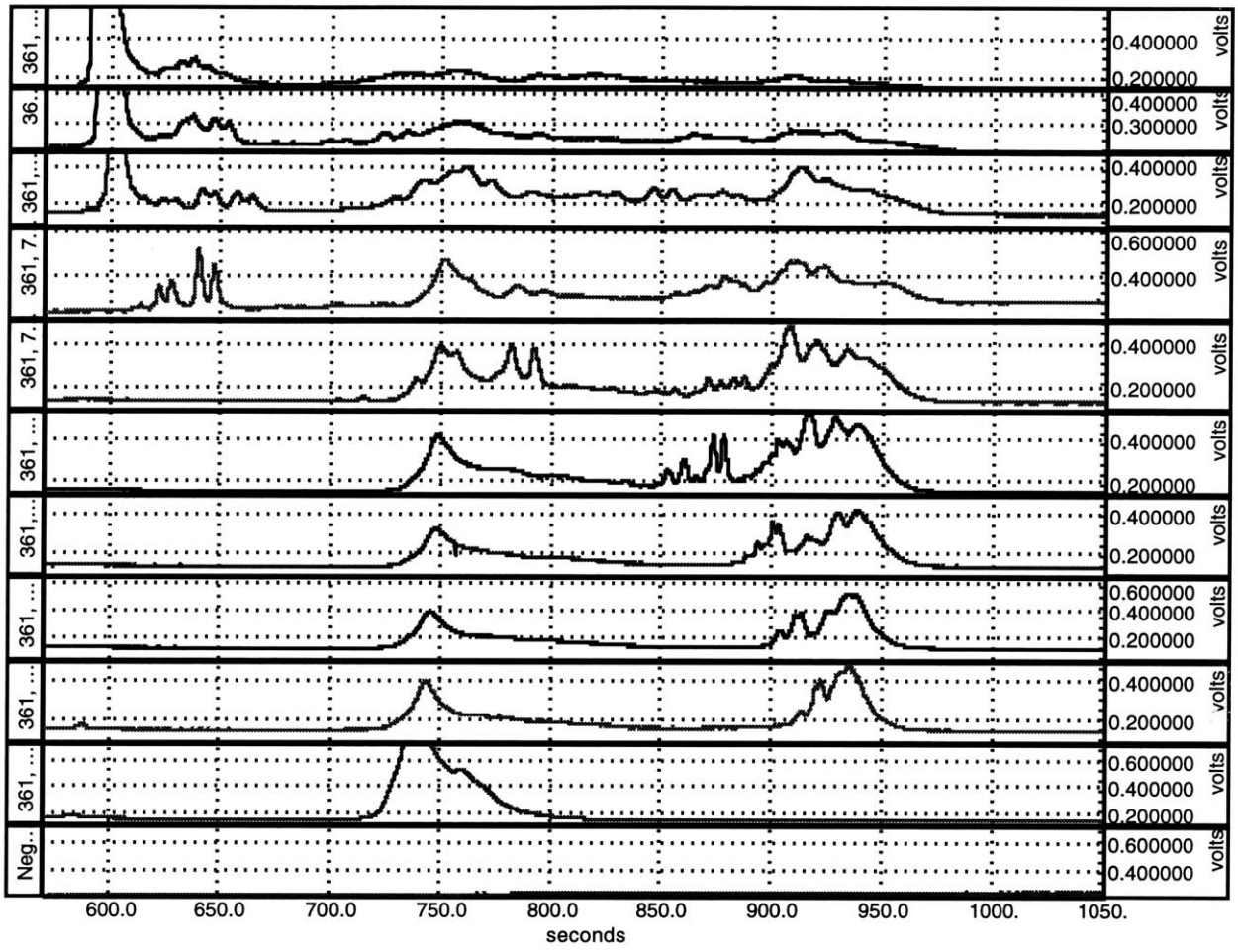

Figure 3.4: Electropherogram of the separation of the 100bp PCR amplicon of the 16S rRNA gene of ASF361(*Lactobacillus sp.*) by CDCE in the temperature range 76-82°C.

These results indicate that the method is itself very sensitive since the different amplicons that the analysis separated into different peaks differed in single nucleotides. This indicates that the goal of studying diversity in ASF species can be achieved by removing the degeneracies of the primers. However, if the region that the primers target has sequence variations between the different ASF species, a different region would have to be chosen for amplification. Such a region has been identified and primers are being designed.

Discussion

The analysis of microbial diversity by using PCR based methods for the 16S rRNA gene is a widespread method, which has been used in ecosystems ranging from hot springs (Barns *et al*, 1994) and soil samples (Bruce *et al*, 1992) to oral microflora (Choi *et al*, 1994). The technique involves the amplification of the 16S rRNA gene from DNA extracted from a microbial community followed by analysis of the PCR products.

The approach taken in this work involves the amplification of a mixture of templates from 8 defined bacterial species followed by the analysis of the amplicons using two alternate strategies, namely terminal restriction fragment length polymorphism (t-RFLP) and constant denaturant capillary electrophoresis (CDCE). The analysis of diversity can be affected by the factors that influence either the PCR step or the subsequent analytical method used.

Although a powerful technique, the amplification of multiple templates leads to potential biases. The bias of primers towards a particular template is one of the biggest pitfalls of this method. The bias is affected by several different factors such as the

sequence identity between the primer and target region, the flanking sequences and the GC content of the template (Reysenbach *et al*, 1992). The result of a bias may be the over-amplification of one of the species and/or inefficient amplification of the others. Further, when one of the templates is present in a much higher copy number than the others, the PCR reaction exhibits a bias towards the formation of products from all templates in a 1:1 ratio. Even in the absence of an excess of one particular template, all products are driven to a 1:1 ratio by saturation kinetics. Thus, one of the products saturates and the rest of them continue to be amplified till they reach saturation. This results in a skewed result that does not in any way reflect the initial ratios of the different templates (Suzuki *et al*, 1996, Polz *et al*, 1998). In mixtures of homologous sequences, chimeric products are often formed by the reannealing of partially amplified templates with a homologous template. These products would have an adverse effect on the analysis that follows the PCR step. A 30% chimeric product generation was seen after the co-amplification of two nearly identical 16S genes (Wang, *et al*, 1996). Further, since a group of diverse bacteria are being amplified, degenerate primers are used and degeneracy takes away any quantitative estimates that could be made from an analysis of the amplicons.

The altered Schaedler flora (ASF) system consists of a diverse group of bacteria and most of the sequences for the 16S gene exhibit a lot of variation, while some of the sequences are similar. All of the above factors are important in the analysis of the ASF system in mice by either of the analytical methods used and should be factored in while analyzing the final results.

The second stage of the method used is the analysis of the mixture of PCR products. The t-RFLP approach involves the differentiation of different species based on the length of the terminal fragment produced by restriction digestion by a chosen set of two enzymes, *HhaI* and *NciI*. The methods consists of three steps: digestion of DNA, purification of digested products and analysis on a capillary electrophoresis machine. Each of these steps is important in terms of interpreting the final result. The choice of restriction enzymes is of prime importance, as this determines the level of separation possible. Since a single enzyme that produced a distinguishable profile for the 8 species could not be found, two enzymes were used. The digestion step has a range of parameters including the temperature of digestion, the duration of digestion, the enzyme concentration and the buffers used. The buffers and temperature are standardized for each enzyme and since both enzymes are active in the same buffer and at the same temperature, these should not be factors of importance. The compatibility of the two enzymes with respect to the buffers and temperature is a very important factor that reduces the variability that would otherwise be inherent for sequential digests with different conditions. When different buffers are used, the activity of one enzyme is affected by the buffer of the other, thus necessitating a further cleaning step, which leads to a loss of yield from each sequential digest. Preliminary results for single species runs indicated that some partially digested products were present. By the above analysis, the factor that caused this was the time of digestion and an increase of the digestion time from 3 hours to overnight digestion helped solve this problem.

Data for mixtures of different species shows the expected peaks, but some other peaks were present. There are several possible reasons for this. Firstly, the peaks could be

carry-overs from low levels of contamination in the initial pure species templates, as the analysis involved the amplification of PCR products. However, visualization of the product on agarose gels did not indicate any extra bands after digestion. This may have been due to the low resolution of agarose gels.

The other possibility is that the extra peaks are chimeric products from the PCR reactions. The 16S rRNA gene exhibits long regions of homology between different species and this increases the possiblity of chimeric species being formed. This can be confirmed by running duplicate reactions and comparing the results. Since chimeric products may be random, the profiles for the extra peaks could be different for different runs.

The run data for Offspring 04/28 samples indicates that there were only 5 peaks on the electrophoretogram. This is less than the expected 8 peaks. Further, the peaks have a sizeable error in fragment length as compared to the expected lengths. A difference in the number of peaks seen can be due to one of two reasons. Firstly, PCR bias, as discussed above, may lead to an increased amplification of some templates. Secondly, all the ASF species may not have been present in the mouse at the same concentrations. This would indicate interaction between the different species within the gut, with the growth of some being favored. Also, the gene copy number for the 16S rRNA gene may be different in the 8 species. A set of experiments is being planned to evaluate this. By varying the concentration of one template while keeping the others constant, bias, if any, can be evaluated. Correlation of CFU data from the same fecal pellets with the t-RFLP data might also help resolve the cause of this observation. The variation of size of the fragments from the expected size might be due to the algorithms used for sizing the peaks

with respect to the GS500 standard. Osborn *et al* (2000) present an analysis where they show that the use of the Local Southern algorithm leads to more accurate results as compared to the third order algorithm used in our analysis. Another possibility for the disparity is machine error. This will be tested out by comparing a large number of runs.

The CDCE technique is based on the differences in melting temperature between short amplicons from the 16S rRNA genes of different species of bacteria. It is an extremely sensitive method which can separate sequences with single base pair polymorphisms (Khrapko *et al*, 1994). The results for a single species run indicate multiple peaks. This was initially thought to be due to polymorphisms at one or two nucleotides in copies of the 16S gene. However, the primers used for amplification were degenerate and this led to the formation of products differing in one or two base pairs, leading to the results seen. The primers are being redesigned so as to avoid this problem.

Thus, the methods used assess the diversity of the ASF species in the samples, but the process has to be optimized further. The future plan for this work involves further standardization of the protocols, with a comparison of different algorithms for sizing in t-RFLP analysis, the evaluation of template bias, the evaluation of the detection limit for the 8 organisms and the analysis of machine inconsistencies, so as to develop a robust method for study of the diversity and ecology of ASF bacteria and the effect of their interactions with different factors. In the long run, the studies will focus on the effects of various insults on the ASF flora in mice and try to evolve an understanding of the ecology of the gut through this simple model.

Appendix A: Alignments

Species	Strain																									
E coli rrsB	K-12	G	G	A	.	G	.	T	.	T	.	C	C	.	.	.	G	A	G	G	C	G	.	G	G	
H pylori	Hp 43504	G	T	T	G	T	T	G	G	A	G	G	G	C	T	T	A	G	T	C	T	C	T	C	C	
H pylori	85D08	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
H pylori	11637	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
H nemestrinae	49396T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
H felis	DS3	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	G	T	C	.	.	.	_	.	.	
H canis	NCTC 12739	.	.	.	.	.	.	.	C	C	C	T	.	.	.	.	G	T	C	A	G	G	_	G	.	
H cinaedi	CCUG 18818	.	.	.	.	.	.	.	C	C	C	T	.	.	.	.	N	T	C	A	G	G	_	G	.	
H cholecystus	Hkb-1	.	.	.	.	.	.	.	.	G	.	T	.	.	.	.	G	T	C	A	C	T	_	.	.	
H salomonis	N.I.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	T	.	.	.	C	.	C	.	.	
H "mainz"	"Mainz"	.	.	.	.	.	.	.	C	C	C	T	.	.	.	.	G	T	C	A	G	G	_	G	.	
F rappini	DBS59	.	.	.	.	.	.	.	C	C	C	T	.	.	.	.	G	T	C	A	G	G	_	G	.	
H bilis	MIT 97-6456	.	.	.	.	.	.	.	C	C	C	T	.	.	.	.	G	T	C	A	G	G	_	G	.	
H pullorum	NCTC 12824	.	.	.	.	.	.	.	T	G	A	.	.	.	.	.	G	T	C	.	.	T	_	G	.	
H trogontum	LRB 8581	.	.	.	.	.	.	.	C	C	C	T	.	.	.	.	G	T	C	A	G	G	_	G	.	
H felis	Eaton 1602	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	G	T	C	.	.	.	_	.	.	
H heilmannii	uncultivated	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	G	T	C	.	.	.	_	.	.	
H muridarum	ATCC 49282	.	.	.	.	.	.	.	C	C	T	T	.	.	.	.	G	A	C	A	G	G	_	G	.	
H rodentium	MIT 95-1707	.	.	.	.	.	.	.	C	G	A	.	.	.	.	.	G	T	C	.	.	T	_	G	.	
H mustelae	ATCC 43772	.	.	.	.	.	.	.	.	G	.	T	.	.	.	.	G	T	C	A	C	T	_	.	.	
H suncus	Kaz-2	.	.	.	.	.	.	.	.	G	.	T	.	.	.	.	_	.	.	.	A	.	_	.	.	
H pametensis	B9A Seymour	.	.	.	.	.	.	.	T	G	.	A	.	.	.	.	G	T	C	T	C	T	_	G	.	
W succinogenes	ATCC 29543	.	.	.	.	.	.	.	C	C	C	T	.	.	.	.	G	T	C	A	G	G	_	G	.	
H hepaticus	Hh-2	.	.	.	.	.	.	.	C	C	T	T	.	.	.	.	G	T	C	A	G	G	_	G	.	

Figure A.1 : Alignment for *Helicobacter pylori* specific primer 787f . The sequence of the primer 787f is 5' GTT GTT GGA GGG CTT AGT CTC TCC 3'. The standard nomenclature for this primer is S-S-H.pylo-0765-a-S-24.

N.I. = No information

Species	Strain																		
E coli rrsB	K-12	.	.	G	.	.	G	A	G	A	A	T	.	.	.	C	.	.	T
H pylori	Hp 43504	G	A	A	A	T	A	G	T	G	G	A	G	T	G	T	C	T	A
H pylori	85D08	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
H pylori	11637	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H nemestrinae	49396T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
H felis	DS3	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.
H canis	NCTC 12739	.	.	G	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.
H cinaedi	CCUG 18818	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	–	.	G
H cholecystus	Hkb-1	.	.	G	.	.	.	.	C	.	.	.	.	.	.	–	.	.	G
H salomonis	N.I.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.
H "mainz"	Mainz	.	.	G	.	.	.	.	C	.	.	.	.	.	.	–	.	.	G
F rappini	DBS59	.	.	G	.	.	.	.	.	.	.	.	.	.	.	–	.	.	G
H bilis	MIT 97-6456	.	.	G	.	.	.	.	.	.	.	.	.	.	.	–	.	.	G
H pullorum	NCTC 12824	.	.	G	.	.	.	.	C	.	.	.	.	.	.	–	.	.	G
H trogontum	LRB 8581	.	.	G	.	.	.	.	C	.	.	.	.	.	.	–	.	–	–
H muridarum	ATCC 49282	.	.	G	.	.	.	T	G	.	.	.	.	.	.	C	.	A	C
H rodentium	MIT 95-1707	.	.	G	.	.	.	.	.	.	.	.	.	.	.	–	.	.	.
H mustelae	ATCC 43772	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.
H suncus	Kaz-2	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H pametensis	B9A Seymour	.	.	G	.	.	.	.	C	.	.	.	.	.	.	C	T	G	G
W succinogenes	ATCC 29543	.	.	G	.	.	.	T	G	.	.	.	.	.	.	C	.	G	G
H hepaticus	Hh-2	.	.	G	.	.	.	.	.	.	.	.	.	.	.	–	.	–	–

Figure A.2 : Alignment for *Helicobacter pylori* specific primer 976f . The sequence of the primer 976f is 5' GAAATAGTGGAGTGTCTAGCTTGCTAGAC 3'. The standard nomenclature for this primer is S-S-H.pylo-0944-a-S-29.
N.I. = No information

Species	Strain																										
E coli rrsB	K-12	.	T	C	G	_	_	A	A	C	C	.	T	G	A	G	A	C	.	G	G	T	G	C	T
H pylori	Hp 43504	T	A	G	C	T	T	G	C	T	A	G	A	C	C	T	T	G	A	A	A	A	C	A	G
H pylori	85D08	.	G	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H pylori	11637	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H nemestrinae	49396T	.	G	.	.	.	.	A	.	.	.	A	.	T	.	C	.	.	.	.	.	.	.	.	.
H felis	DS3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.
H canis	NCTC 12739	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H cinaedi	CCUG 18818	.	G	.	.	.	.	.	.	C	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.
H cholecystus	Hkb-1	.	G	.	.	.	.	.	.	C	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.
H salomonis	N.I.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.
H "mainz"	"Mainz"	.	G	.	T	.	.	.	.	C	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.
F rappini	DBS59	.	G	.	.	.	.	.	.	C	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.
H bilis	MIT 97-6456	.	G	.	.	.	.	.	.	C	.	.	.	G	.	.	.	.	.	.	N	.	.	.	.
H pullorum	NCTC 12824	.	G	.	.	.	.	.	.	C	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.
H trogontum	LRB 8581	_	_	C	.	.	.	C	G	G	G	.	.	G	.	.	.	.	.	.	.	.	.	.	.
H felis	Eaton 1602	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.
H heilmannii	uncultivated	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.
H muridarum	ATCC 49282	.	C	_	_	.	.	C	T	G	T	.	A	G	.	T	.	G	A	.	.	.	.	.	.
H rodentium	MIT 95-1707	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.
H mustelae	ATCC 43772	.	.	.	T	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H suncus	Kaz-2	.	.	.	T	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H pametensis	B9A Seymour	.	G	_	.	.	.	.	.	C	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.
W succinogenes	ATCC 29543	.	G	_	T	.	.	A	.	.	G	.	.	G	.	.	.	.	.	.	.	.	.	.	.
H hepaticus	Hh-2	_	_	_	.	.	.	C	G	G	G	.	.	G	.	.	.	.	.	.	.	.	.	.	.

Figure A.3 : Alignment for *Helicobacter pylori* specific primer 1017r . The sequence of the primer 1017r is 5'ACCTGTTTTCAAGGTCTAGCAAGCTA 3'. The standard nomenclature for this primer is S-S-H-pylo-0960-a-A-26.
N.I. = No information

Species	Strain	
E coli rrsB	K-12	_ T . C . . _ _ _ _ _ . C . G . . . . . . A . . . . . G . .
H pylori	Hp 43504	A A C A G G T T A T G C T G A G A A C T C T A A G G A T A C
H pylori	85D08	. . . . C . . . .
H pylori	11637	. . . .
H nemestrinae	49396T	. . . . . . _ C . . . .
H felis	DS3	. . . . N . . . . C T . . . . A . . . .
H canis	NCTC 12739	. G . . . _ . . C G . . . . . . C . . . . G . .
H cinaedi	CCUG 18818	. G . . . _ C . C G . . . . . . C . . . . G . .
H cholecystus	Hkb-1	. G . . . _ . . C G . . . . . . C . . . . . . G . . . G . .
H salomonis	N.I.	. . . . G . . . . C T . . . . A . . . .
H "mainz"	"Mainz"	. G . . . _ . . C G . . . . . . C . . . . G . .
F rappini	DBS59	. G . . . _ . . C G . . . . . . C . . . . G . .
H bilis	MIT 97-6456	. G . . . _ . . C G . . . . . . C . . . . G . .
H pullorum	NCTC 12824	. G . . . _ . . C G . . . . . . C . . . . G . .
H trogontum	LRB 8581	. G . . . _ . . C G . . . . . . C . . . . G . .
H felis	Eaton 1602	. . . . N N . . . . C T . . . . A . . . .
H heilmannii	uncultivated	. . . . R . . . . C T . . . . A . G . .
H muridarum	ATCC 49282	. G . . . T . . N A . . . . . . C . . . . G . .
H rodentium	MIT 95-1707	. . . . . _ . . C G . T A . . . C . . . . G . .
H mustelae	ATCC 43772	. G . T A _ . . C G . . . . . . C . . . . A . G . .
H suncus	Kaz-2	. G . . . T . . C G . . . . . . C . . . . A . G . .
H pametensis	B9A Seymour	. . . . . _ . . C G . . . . . . C . . . . . . G . . . G . .
W succinogenes	ATCC 29543	. . . . . _ . . C G . . C . . . C . . . . . . G T . . G . .

Figure A.4 : Alignment for *Helicobacter pylori* specific primer 1127r. The sequence of the primer 1127r is 5' GGTA TCC TTA GAG TTC TCA GCA TAA CCT GTT 3'. The standard nomenclature for this primer is S-S-H.pylo-1064-a-A-30.
N.I. = No information

Species	Strain																														
E coli rrsB	K-12	.	.	.	G	T	.	.	.	C	.	G	A	.	G	G	G	.	.	T	.	.	.	.	A	.	.	A
H pylori	Hp 43504	G	A	T	C	A	G	C	C	T	A	T	G	T	C	C	T	A	T	C	A	G	C	T	T	G	T	T
H pylori	85D08	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H pylori	11637	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H nemestrinae	49396T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H felis	DS3	.	.	.	T	G	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H canis	NCTC 12739	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H cinaedi	CCUG 18818	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H cholecystus	Hkb-1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H salomonis	N.I.	.	.	.	T	G	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H "mainz"	"Mainz"	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H bilis	MIT 95-1707	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H pullorum	NCTC 12824	.	.	.	T	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H trogontum	LRB 8581	.	.	.	T	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H felis	Eaton 1602	.	.	.	T	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H heilmannii	uncultivated	.	.	.	T	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H muridarum	ATCC 49282	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H rodentium	MIT 95-1707	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H mustelae	ATCC 43772	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.
H suncus	Kaz-2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H pametensis	B9A Seymour	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
W succinogenes	ATCC 29543	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H hepaticus	Hh-2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Figure A.5 : Alignment for *Helicobacter* genus specific primer 212f. The primer sequence is 5' GAT CAG CCT ATG TCC TAT CAG CTT GTT 3'. The standard nomenclature for this primer is S-G-Heli-0192-a-S-27.

N.I. = No information

Species	Strain																								
E coli rrsB	K-12	T	C	C	C	.	.	G	.	T	.	.	T	.	.	.	.	.	.	.	.	.	A	.
H pylori	Hp 43504	C	G	G	G	T	A	T	C	C	G	G	C	C	T	G	A	G	A	G	G	G	T
H pylori	85D08	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H pylori	11637	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H nemestrinae	49396T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H felis	DS3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H canis	NCTC 12739	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H cinaedi	CCUG 18818	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H cholecystus	Hkb-1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H salomonis	N.I.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H "mainz"	"Mainz"	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
F rappini	DBS59	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H bilis	MIT 97-6456	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H pullorum	NCTC 12824	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H trogontum	LRB 8581	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H felis	Eaton 1602	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H heilmannii	uncultivated	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H muridarum	ATCC 49282	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H rodentium	MIT 95-1707	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H mustelae	ATCC 43772	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.
H suncus	Kaz-2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H pametensis	B9A Seymour	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
W succinogenes	ATCC 29543	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H hepaticus	Hh-2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Figure A.6 : Alignment for *Helicobacter* genus specific primer 289r. The primer sequence is 5' ACC CTC TCA GGC CGG ATA CCC G 3'. The standard nomenclature for this primer is S-G-Heli-0248-a-A-22.
N.I. = No information

Species	Strain																					
E coli rrsB	K-12	C	.	G	G	T	G	C	T	.	C	A	T	G	.	.	T	G	T	C	G
H pylori	Hp 43504	G	A	A	A	A	C	A	G	G	T	G	C	T	G	C	A	C	G	G	C
H pylori	85D08	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H pylori	11637	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H nemestrinae	49396T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H felis	DS3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H canis	NCTC 12739	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H cinaedi	CCUG 18818	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.
H cholecystus	Hkb-1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H salomonis	N.I.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H "mainz"	"Mainz"	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
F rappini	DBS59	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H bilis	MIT 97-6456	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H pullorum	NCTC 12824	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H trogontum	LRB 8581	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H felis	Eaton 1602	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H heilmannii	uncultivated	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H muridarum	ATCC 49282	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H rodentium	MIT 95-1707	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H mustelae	ATCC 43772	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H suncus	Kaz-2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H pametensis	B9A Seymour	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
W succinogenes	ATCC 29543	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H hepaticus	Hh-2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Figure A.7 : Alignment for *Helicobacter* genus specific primer 1026r. The primer sequence is 5' GCC GTG CAG CAC CTG TTT TC 3'. The standard nomenclature for this primer is S-G-Heli-0974-a-A-20.
N.I. = No information

Appendix B

Sensitivity calculations for *Helicobacter pylori* specific primer pair 787f-1127r

DNA conc. = 19ng/µl

Total DNA/reaction = 38ng

Mol. Wt. Of average base pair = 660g/mole

Assumed length of H. pylori genome = 1.65 Mb

$$\text{Wt./copy of genome} = \frac{1.65 \times 10^6 \times 660}{6 \times 10^{23}}\ \text{g} = 1.82 \times 10^{-15}\ \text{g}$$

$$\text{No of copies in 38ng} = \frac{38 \times 10^{-9}}{6 \times 10^{23}} = 2 \times 10^7 \text{ copies}$$

Copies of SSU rrn operon in H. pylori = 2 (Tomb *et al*, 1997)

$\therefore$ total copies of 16S gene in 38ng of template DNA = 4×10^7

For 787f-1127r in DNA from fecal pellets,

Detection limit = 10^{-6} dilution = $10^{-6} \times 4 \times 10^7$

= 40 copies

= 20 bacteria

Appendix C: References

Alm RA, Ling LS, Moir DT, King BL, Brown ED, Doig PC, Smith DR, Noonan B, Guild BC, deJonge BL, Carmel G, Tummino PJ, Caruso A, Uria-Nickelsen M, Mills DM, Ives C, Gibson R, Merberg D, Mills SD, Jiang Q, Taylor DE, Vovis GF, Trust TJ. Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. Nature. 1999 Jan 14;**397**(6715):176-80.

Avaniss-Aghajani E, Jones K, Holtzman A, Aronson T, Glover N, Boian M, Froman S, Brunk CF. Molecular technique for rapid identification of mycobacteria. J Clin Microbiol. 1996 Jan;**34**(1):98-102.

Barns SM, Fundyga RE, Jeffries MW, Pace NR. Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. Proc Natl Acad Sci U S A. 1994 Mar 1; **91**(5):1609-13.

Blaser MJ. *Helicobacter pylori* and the pathogenesis of gastroduodenal inflammation. J Infect Dis. 1990 Apr;**161**(4):626-33. Review.

Bruce KD, Hiorns WD, Hobman JL, Osborn AM, Strike P, Ritchie DA. Amplification of DNA from native populations of soil bacteria by using the polymerase chain reaction. Appl Environ Microbiol. 1992 Oct; **58**(10):3413-6.

Choi BK, Paster BJ, Dewhirst FE, Gobel UB. Diversity of cultivable and uncultivable oral spirochetes from a patient with severe destructive periodontitis. Infect Immun. 1994 May; **62**(5):1889-95.

Chong SK, Lou Q, Fitzgerald JF, Lee CH. Evaluation of 16S rRNA gene PCR with primers Hp1 and Hp2 for detection of *Helicobacter pylori.* J Clin Microbiol. 1996 Nov;**34**(11):2728-30.

Cotran, Kumar and Robbins. Robbins' pathological basis of disease, 1999

Covacci,A., Telford,J.L., Giudice,G.D., Parsonnet,J. Rappuoli, R. *Helicobacter pylori* Virulence and Genetic Geography. Science 1999 May 21; **284**: 1328-1333.

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

Dewhirst FE, Chien CC, Paster BJ, Ericson RL, Orcutt RP, Schauer DB, Fox JG. Phylogeny of the defined murine microbiota: altered Schaedler flora. Appl Environ Microbiol. 1999 Aug;**65**(8):3287-92.

Doig P, de Jonge BL, Alm RA, Brown ED, Uria-Nickelsen M, Noonan B, Mills SD, Tummino P, Carmel G, Guild BC, Moir DT, Vovis GF, Trust TJ. *Helicobacter pylori* physiology predicted from genomic comparison of two strains. Microbiol Mol Biol Rev. 1999 Sep;**63**(3):675-707. Review.

Dunn BE, Cohen H, Blaser MJ. *Helicobacter pylori.* Clin Microbiol Rev. 1997 Oct;**10**(4):720-41. Review.

Eaton KA, Brooks CL, Morgan DR, Krakowka S. Essential role of urease in pathogenesis of gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. Infect Immun. 1991 Jul;**59**(7):2470-5.

Enroth H, Engstrand L. Immunomagnetic separation and PCR for detection of *Helicobacter pylori* in water and stool specimens. J Clin Microbiol. 1995 Aug;**33**(8):2162-5.

Fox JG, Batchelder M, Marini R, Yan L, Handt L, Li X, Shames B, Hayward A, Campbell J, Murphy JC. *Helicobacter pylori*-induced gastritis in the domestic cat. Infect Immun. 1995 Jul;**63**(7):2674-81.

Genta RM, Robason GO, Graham DY. Simultaneous visualization of *Helicobacter pylori* and gastric morphology: a new stain. Hum Pathol. 1994 Mar;**25**(3):221-6.

Go MF, Kapur V, Graham DY, Musser JM. Population genetic analysis of *Helicobacter pylori* by multilocus enzyme electrophoresis: extensive allelic diversity and recombinationalpopulation structure. J Bacteriol,1996;**178**:3934-8.

Goodwin CS, McCulloch RK, Armstrong JA, Wee SH. Unusual cellular fatty acids and distinctive ultrastructure in a new spiral bacterium (Campylobacter pyloridis) from the human gastric mucosa. J Med Microbiol. 1985 Apr;**19**(2):257-67.

Goodwin, C.S. (ed) *Helicobacter pylori* : biology and clinical practice, CRC press, 1993.

Grubel P, Huang L, Masubuchi N, Stutzenberger FJ, Cave DR. Detection of *Helicobacter pylori* DNA in houseflies (Musca domestica) on three continents. Lancet. 1998 Sep 5;**352**(9130):788-9.

Gutell RR, Larsen N, Woese CR. Lessons from an evolving rRNA: 16S and 23S rRNA structures from a comparative perspective. Microbiol Rev. 1994 Mar;**58**(1):10-26. Review.

Handt LK, Fox JG, Stalis IH, Rufo R, Lee G, Linn J, Li X, Kleanthous H. Characterization of feline *Helicobacter pylori* strains and associated gastritis in a colony of domestic cats. J Clin Microbiol. 1995 Sep;**33**(9):2280-9.

Hirayama F, Takagi S, Kusuhara H, Iwao E, Yokoyama Y, Ikeda Y. Induction of gastric ulcer and intestinal metaplasia in mongolian gerbils infected with *Helicobacter pylori*. J Gastroenterol. 1996 Oct;**31**(5):755-7.

Ho SA, Hoyle JA, Lewis FA, Secker AD, Cross D, Mapstone NP, Dixon MF, Wyatt JI, Tompkins DS, Taylor GR, et al. Direct polymerase chain reaction test for detection of *Helicobacter pylori* in humans and animals. J Clin Microbiol. 1991 Nov;**29**(11):2543-9.

Hulten K, Han SW, Enroth H, Klein PD, Opekun AR, Gilman RH, Evans DG, Engstrand L, Graham DY, El-Zaatari FA. *Helicobacter pylori* in the drinking water in Peru. Gastroenterology. 1996 Apr;**110**(4):1031-5.

Karita M, Kouchiyama T, Okita K, Nakazawa T. New small animal model for human gastric *Helicobacter pylori* infection: success in both nude and euthymic mice. Am J Gastroenterol. 1991 Nov;**86**(11):1596-603.

Khrapko K, Hanekamp JS, Thilly WG, Belenkii A, Foret F, Karger BL. Constant denaturant capillary electrophoresis (CDCE): a high resolution approach to mutational analysis. Nucleic Acids Res. 1994 Feb 11;**22**(3):364-9.

Liu WT, Marsh TL, Cheng H, Forney LJ. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl Environ Microbiol. 1997 Nov;**63**(11):4516-22.

Madsen KL, Doyle JS, Jewell LD, Tavernini MM, Fedorak RN. Lactobacillus species prevents colitis in interleukin 10 gene-deficient mice. Gastroenterology. 1999 May;**116**(5):1107-14.

Marchetti M, Arico B, Burroni D, Figura N, Rappuoli R, Ghiara P. Development of a mouse model of *Helicobacter pylori* infection that mimics human disease. Science. 1995 Mar 17;**267**(5204):1655-8.

Marsh TL. Terminal restriction fragment length polymorphism (T-RFLP): an emerging method for characterizing diversity among homologous populations of amplification products. Curr Opin Microbiol. 1999 Jun;2(3):323-7. Review.

Marshall BJ, Armstrong JA, McGechie DB, Glancy RJ. Attempt to fulfil Koch's postulates for pyloric Campylobacter. Med J Aust. 1985 Apr 15;**142**(8):436-9.

Marshall BJ, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet. 1984 Jun 16;**1**(8390):1311-5.

Miehlke S, Thomas R, Guiterrez O, Graham DY, Go MF. DNA fingerprinting of single colonies of *Helicobacter pylori* from gastric cancer patients suggests infection with a single predominant strain. J Clin Microbiol. 1999 Jan;**37**(1):245-7.

Mohammadi M, Redline R, Nedrud J, Czinn S. Role of the host in pathogenesis of Helicobacter-associated gastritis: H. felis infection of inbred and congenic mouse strains. Infect Immun. 1996 Jan;**64**(1):238-45.

Monti J, Fay M, Banchio C, Amendola R, Farias R, Musi A, Sanchez A, Kesner L, Katz J, Ferro F, Kogan Z, Corti R. Detection of *Helicobacter pylori* by polymerase reaction in bile samples from gallbladder and bile stones. Acta Gastroenterol Latinoam. 1999;**29**(4):251-3.

Morotomi M, Hoshina S, Green P, Neu HC, LoGerfo P, Watanabe I, Mutai M, Weinstein IB. Oligonucleotide probe for detection and identification of Campylobacter pylori. J Clin Microbiol. 1989 Dec;**27**(12):2652-5.

Orcutt, R.P., Gianni, F.J., and Judge, R.J. 1987. Development of an "Altered Schaedler Flora" for NCI gnotobiotic rodents. Microecol. Theor. **17**: 59

Polz MF, Cavanaugh CM. Bias in template-to-product ratios in multitemplate PCR. Appl Environ Microbiol. 1998 Oct; **64**(10):3724-30.

Reysenbach AL, Giver LJ, Wickham GS, Pace NR. Differential amplification of rRNA genes by polymerase chain reaction. Appl Environ Microbiol. 1992 Oct; **58**(10):3417-8.

Riggio MP, Lennon A. Identification by PCR of *Helicobacter pylori* in subgingival plaque of adult periodontitis patients. J Med Microbiol. 1999 Mar;**48**(3):317-22.

Romaniuk PJ, Zoltowska B, Trust TJ, Lane DJ, Olsen GJ, Pace NR, Stahl DA. Campylobacter pylori, J Bacteriol. 1987 May;**169**(5):2137-41.

Smith JG, Kong L, Abruzzo GK, Gill CJ, Flattery AM, Scott PM, Bramhill D, Cioffe C, Thompson CM, Bartizal K. PCR detection of colonization by *Helicobacter pylori* in conventional, euthymic mice based on the 16S ribosomal gene sequence. Clin Diagn Lab Immunol. 1996 Jan;**3**(1):66-72.

Smoot DT, Mobley HL, Chippendale GR, Lewison JF, Resau JH. *Helicobacter pylori* urease activity is toxic to human gastric epithelial cells. Infect Immun. 1990 Jun;**58**(6):1992-4.

Suau A, Bonnet R, Sutren M, Godon JJ, Gibson GR, Collins MD, Dore J. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. Appl Environ Microbiol. 1999 Nov;**65**(11):4799-807.

Suzuki MT, Giovannoni SJ. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl Environ Microbiol. 1996 Feb;62(2):625-30.

Tomb JF, White O, Kerlavage AR, Clayton RA, Sutton GG, Fleischmann RD, Ketchum KA, Klenk HP, Gill S, Dougherty BA, Nelson K, Quackenbush J, Zhou L, Kirkness EF, Peterson S, Loftus B, Richardson D, Dodson R, Khalak HG, Glodek A, McKenney K, Fitzegerald LM, Lee N, Adams MD, Venter JC, et al. The complete genome sequence of the gastric pathogen *Helicobacter pylori.* Nature. 1997 Aug 7;**388**(6642):539-47.

Wang GC, Wang Y. The frequency of chimeric molecules as a consequence of PCR co-amplification of 16S rRNA genes from different bacterial species. Microbiology. 1996 May;**142 (Pt 5)**:1107-14.