

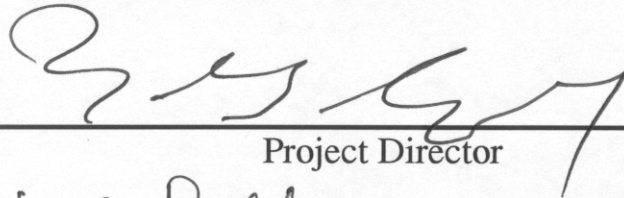
Construction and Characterization of *hypC* Mutants in
Helicobacter pylori

by
Rachael Davis

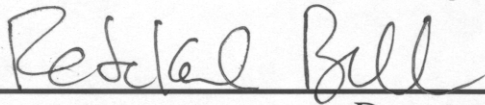
Project Director: Dr. Henry Spratt
Examination Date: November 14, 2002

Rebekah P. Bell
Dr. J. Hill Craddock
Dr. Edward Rozema

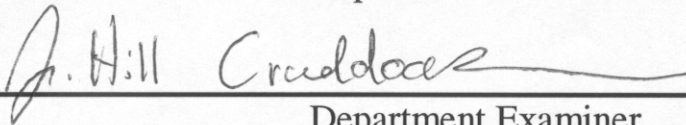
Examining Committee Signatures:



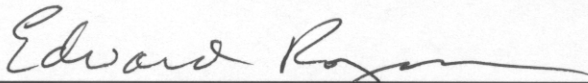
Project Director



Department Examiner



Department Examiner



Liaison, Departmental Honors Committee



Chairperson, University Departmental Honors Committee

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Abstract

Helicobacter pylori is a spiral bacterium that can cause gastritis, ulcers, and cancer in humans. Activity of the enzyme urease is one factor that allows this organism to colonize within the gastric environment. Several structural and accessory proteins have been shown to be required for full activity of this enzyme. The purpose of this study was to investigate the role of the accessory protein encoded for by the *hypC* gene in urease activity. Four mutants that did not produce the accessory protein were obtained by interrupting the *hypC* gene with a kanamycin resistance cassette. These mutants were then assayed to determine if they were affected for urease activity. Under normal growth conditions, all mutants obtained were shown to have diminished urease activity levels when compared to wild type *H. pylori* strain 43504. The results of this study show the accessory protein encoded for by the *hypC* gene is required for the complete function of the enzyme urease.

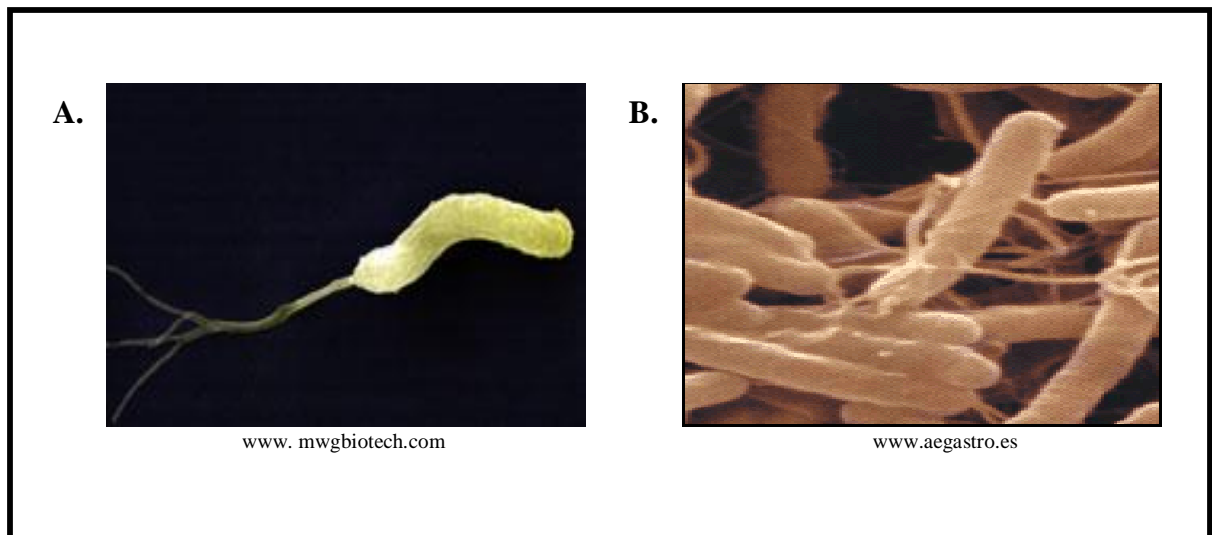
Introduction and Background

Helicobacter pylori is a spiral, gram-negative bacterium that colonizes the gastric mucosa of humans. After colonization, it can persist for many years within the host, where it can cause chronic gastritis as well as gastric and duodenal ulcers. Mucosa Associated Lymphoid Tissue (MALT) lymphoma and adenocarcinomas of the stomach have also been attributed to prolonged infection of this organism (1, 2). Although it is one of the most common bacterial pathogens in the world, mode of transmission of *H. pylori* is still unknown. Transmission probably occurs through contact with contaminated feces, saliva, vomit, or water (3).

The study of *H. pylori* falls relatively recently on the overall timeline of the study of microbial organisms, as it was first isolated in 1982 by Dr. Barry J. Marshall in Perth, Australia (4). Before its discovery, ulcers were thought to be triggered by stress, spicy food, and hypersecretion of acid within the stomach. Now, nine out of ten ulcers are attributed to *H. pylori* infection (5).

Morphologically, the organism is known to grow 2.5 to 5 μm long, and 0.5 to 1.0 μm wide. It can possess from two to seven unipolar, sheathed flagella (Figure 1). Optimal growth occurs in a microaerophilic, or reduced oxygen, environment. In the laboratory, it can be cultured on media supplemented with blood grown in gas jars or CO_2 incubators at 37° Celsius (1).

Figure 1, A and B: Morphology of *Helicobacter pylori*



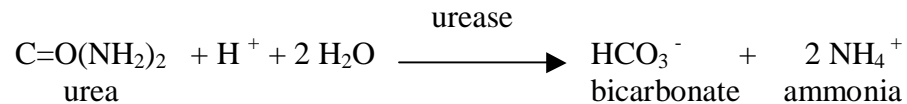
Epidemiological studies have shown that the majority of *H. pylori* infections occur in developing countries, usually in children of lower socioeconomic classes (1). It is also prevalent among lower income adults that live in crowded conditions with poor sanitation. In developed countries, there is a low occurrence of infection among people under the age of forty. In countries that have progressively become more developed over the last fifty years, a marked decline in rate of ulcer development is observed as the economy becomes more affluent and sanitation conditions improve. The development of Japan is a good example of this. Here, more than 50% of persons born before 1960 are infected, while today less than 5% of children acquire the bacterium (1). Results of these studies seem to support the proposed mode of transmission, as people who live in countries where they are more likely to encounter contaminated water and feces show higher rates of infection.

According to the Centers for Disease Control, there are several methods for the diagnosis and treatment of *H. pylori* infections. Diagnosis can be made through both invasive procedures, such as the use of endoscopy to obtain biopsies of gastric tissue, and noninvasive procedures. Noninvasive testing can be in the form of serological tests for *H. pylori* specific antibodies, or by breath tests. Here, patients drink a liquid containing ^{13}C or ^{14}C -labeled urea, which can be broken down into carbon dioxide by the bacterium. The presence of an infection is determined by the occurrence of labeled carbon in the exhaled breath of patients.

Once diagnosed, patients may be treated by antibiotic drug therapy. Antibiotics such as amoxicillin, clarithromycin, or, in adults, tetracycline may be used in conjunction with ranitidine bismuth citrate or a proton pump inhibitor to effectively stop bacterial growth. Treatment is usually given over the course of one to two weeks, and is effective. Only 6% of patients have recurrent ulcers after antibiotic therapy (5).

The ability to colonize, persist, and cause disease in the acidic environment of the stomach is aided by several factors. The presence of sheathed flagella allows the organism to move in a spiral motion. This movement is thought to aid in the penetration of the gastric mucosa by allowing it to burrow into the lining with a screw-like manner. Experiments with animal models have demonstrated that full motility is an essential virulence factor for *H. pylori* by showing the inability of nonmotile mutants to colonize within mice and gnotobiotic pigs (6, 7).

Another factor aiding in colonization is the production of the metalloenzyme urease. Within the stomach, this enzyme converts the small amounts of urea naturally found in saliva and gastric juices into ammonia and bicarbonate in the following reaction:



Here, urea combines with hydrogen in the stomach acid immediately surrounding the cell. Hydrogen is used in the formation of products, and is thus removed from the acid. The products formed are also strong bases, which function to neutralize acids. Therefore, there is a net increase in pH around the cell. The urease reaction seemingly functions to buffer the cell against the harsh acidic climate of the stomach, and appears to facilitate cell survival until penetration of the mucosa occurs (8).

Seven structural genes, known as the *ure* genes, are necessary for the synthesis of an active urease enzyme. *ureA* and *ureB* encode for the structural subunits of urease, while *ureE*, *-F*, *-G*, and *-H*, encode for accessory proteins that aid in the insertion of nickel into the active site of the enzyme. It is this requirement of nickel that makes the enzyme a metalloenzyme. Finally, the gene *ureI* encodes for a membrane protein that functions in urea uptake by the cell (9).

Urease is located throughout the cytoplasm of the cell, and is found in small quantities on the cell surface of *H. pylori*. Weeks *et al* (2000) have shown that it is the cytoplasmic urease that is responsible for the generation of ammonia by bacteria attempting to colonize the stomach. The urea transport protein encoded for by *ureI* is

located in the inner membrane of the cell. It is responsible for the increased rate of urea uptake by the cytoplasm when the cell is in an acidic environment. Urea is then broken down into ammonia, which acts as a buffer for the periplasm surrounding the cell. This buffering allows the pH of the cell to stay between the optimal survival range of 4.0 and 8.5, even though it is located in the pH 2 environment of the stomach. Thus, it is the activity of urease that allows the cell to survive and colonize within the stomach (9).

More recent experiments have shown that accessory proteins encoded for by other genes in *H. pylori* must also be present for full activity of urease. Experiments by Olson and coworkers (2001) have shown that accessory proteins related to hydrogenase, another metalloenzyme, are required in addition to accessory proteins encoded for by the *ure* genes for urease activity to occur (10). Hydrogenase is encoded for by the *hyp* genes, and functions to breakdown molecular hydrogen, H₂, into two hydrogen ions and two electrons for use during energy metabolism (11).

During experiments by Olson *et al* (2001), mutants were made for *hypA*, *hypB*, *hypD*, and *hypF* genes by inserting a kanamycin resistance cassette (kan^R) into the gene. Kanamycin is an antibiotic, an antimicrobial agent naturally produced in a microorganism that inhibits or kills other microorganisms. It belongs to the chemical class of antibiotics known as aminoglycosides, which inhibit protein synthesis in both gram-positive and gram-negative cells (12). Kanamycin specifically binds to the 70 S subunit of ribosomes in these cells and promotes misreading of mRNA during

translation (13). Therefore, functioning proteins are not formed, and cellular processes are inhibited.

Microorganisms have successfully developed resistance to various antibiotics, including kanamycin. The gene for kanamycin resistance (Appendix A) can be found naturally within the chromosome of a microbe, or within the extra chromosomal DNA found outside of the nucleus known as a plasmid. Within a cell, this gene encodes for the protein aminoglycoside phosphotransferase (12). This enzyme functions to inactivate the antimicrobial action of kanamycin by transferring a phosphoryl group from ATP to the aminoglycoside structure. This inactivates the molecule, and prevents binding to the ribosomal subunit (14). Therefore, the antibiotic does not harm the cell.

If it does not occur naturally within a bacterium, the kanamycin resistance gene can be inserted into a specific DNA sequence through genetic manipulation, as seen in the Olson project. The gene sequence, which is also called a cassette, was used in this project to disrupt the function of the *hyp* genes. When inserted into the genome, the kan^R disrupts the existing nucleotide sequence. Thus, the correct nucleotide sequence to ensure the synthesis of active proteins is not available, and the accessory proteins normally encoded for by these genes are not produced. Aminoglycoside phosphotranferase is produced, so the cells develop a resistance to kanamycin.

Once the kan^R was successfully incorporated into the *hypA*, *hypB*, *hypD*, and *hypF* genes, all mutants observed were shown to be hydrogenase negative when

tested, as is expected. Surprisingly, mutants for the *hypA* and *hypB* genes were also affected for urease activity as well. Urease activity in these strains was observed to be 200-fold and 40-fold times lower, respectively, than that of the wild type strain of *H. pylori*. Although some progress has been made in better understanding the roles of accessory proteins in the maturation of metalloenzymes, the specific steps are still unknown (10, 15). The Olson experiments were exciting in that they clearly showed a link between the pathways of accessory proteins for two different metalloenzymes.

During these previous attempts to construct mutants for *hyp* genes, no mutant could be obtained for the *hypC* gene due to the lack of a convenient restriction site for the insertion of the kanamycin cassette. The purpose of this project was to construct a mutant for *hypC* by introducing a restriction site into the gene, and then inserting a kanamycin resistance cassette to disrupt the gene function. A restriction site is a sequence of nucleotides within the DNA that are recognized by a restriction endonuclease. This enzyme cleaves the bonds between the nucleotides at a specific point, creating a break in the gene sequence where a fragment of DNA can be inserted. The insertion of the kan^R disrupts the gene sequence, and prevents the replication of a fully functioning *hypC* gene. Therefore, no accessory protein is encoded for by the gene. Because the genome of *H. pylori* has been fully sequenced, *hypC* is known to encode for a protein designated HypC. This protein is 77 amino acids in length, and has a molecular weight of 8627 Daltons (16, 17). It is thought to be a chaperone like protein that is involved in the incorporation of nickel into the

hydrogenase binding site. It may also be required for full urease activity within the cell.

Once cells fully incorporated the kan^R into the *hypC* gene, the resulting mutants were assayed to determine they are affected for hydrogenase and urease activity. The results of these assays could possibly compliment the Olson *et al* project, and could possibly lead to a better understanding of the role of accessory proteins in the maturation and functioning of metalloenzymes in *H. pylori*.

Methods and Materials

Bacterial Strains and Growth Conditions. *H. pylori* American Tissue Culture Collection (ATCC) 43504 was used as the wild type strain. Cultures were grown on Brucella agar (DIFCO) supplemented with 10% defibrinated sheep blood (Gibson Laboratories, Inc.). These are referred to as BA plates. Kanamycin (at 30 µg/ml) was added to the media in order to culture only those cells that had successfully incorporated the kanamycin resistance cassette into their genome. Cells were grown in a CampyPak Plus (Becton Dickinson) environment at 37 °C in 4% oxygen for three days (10). Genetic manipulations were performed in *Escherchia coli* strain DH5α grown in Luria Broth or on Luria agar (DIFCO) plates supplemented with ampicillin (at 100 µg/ml).

Insertion of *EcoRV* Restriction Site Using Overlapping PCR. Genomic DNA from *H. pylori* 43504 was extracted using the Quantum Prep Aquapure Genomic DNA Kit (Quantum). In this process, the cellular membrane was ruptured using an alkaline lysis buffer. All of the contents of the cell, with the exception of DNA, were degraded using enzymes. DNA was filtered out and suspended in water. A sample of this DNA was then amplified using polymerase chain reaction, or PCR. This process, developed by scientist Kary Mullis in the 1980's, can produce millions of copies of a piece of DNA in a short amount of time. PCR occurs in three steps - denaturing, annealing, and elongation. During denaturization, the DNA to be amplified is heated at a high temperature. This weakens the bonds between the 5' and 3' strands, and causes them to break. This effectively "unzips" the molecule, and divides the DNA into two single strands. Primers then bind to the single strands in the annealing process. These are short chains of nucleotides that are complimentary to a specific nucleotide sequence in the DNA. The primers bind to the strands with the help of the DNA polymerase, an enzyme that matches complimentary base pairs together. The temperature is lowered in this step to allow bonds to form between the strands. In the final step of PCR, elongation, polymerase begins synthesizing more 5' and 3' strands of the DNA by filling in the sequences after the primer with free floating nucleotides that have been added to the mixture. The chains of nucleotides created are the same as the original piece of DNA.

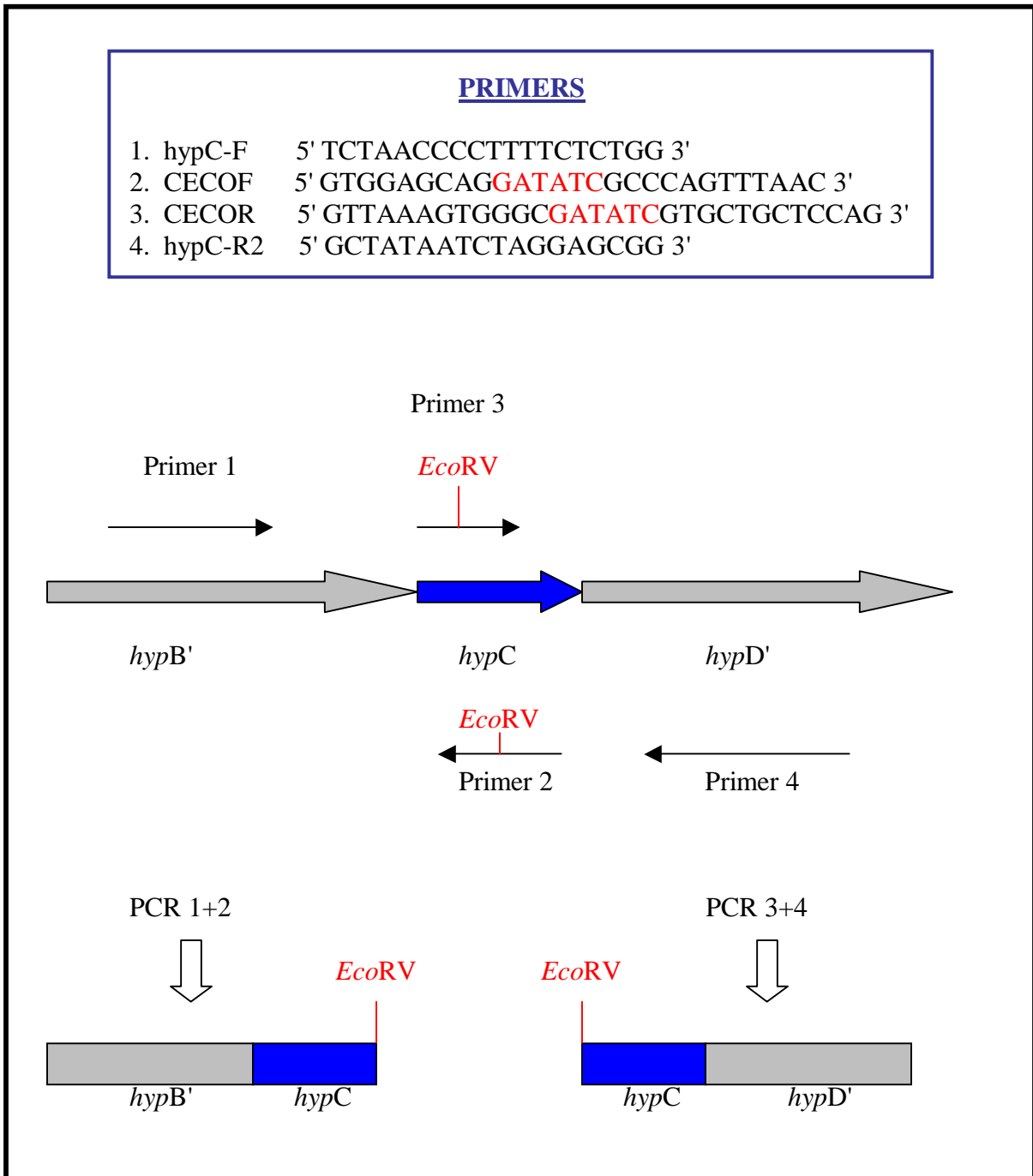
For this experiment, the DNA sequences from *hypC*, as well as *hypB* and *hypD*, were chosen as the genes to be amplified. The length of the *hypC* gene is

rather small, as it is only 233 base pairs long (16). Portions of these surrounding genes were amplified in order to allow chromosomal recombination to occur when the kan^R was inserted. Recombination usually requires at least 200 bases flanking each side of the cassette. The sequence of the DNA for the *hyp* genes is listed in Appendix B.

Four primers were chosen to correspond to four different nucleotide sequences within this fragment of DNA (Figure 2). All were purchased from Integrated DNA Technologies, Inc., Coralville, IA. Primer 1 was created for use in amplifying *hyp* genes during the Olson experiment, and was given the name hyp C-F. It is 19 bases long, and anneals to the 5' strand of the *hypB* gene of the denatured DNA. Primer 4 is named hyp C-R2, and is 18 bases in length. It anneals to the 3' strand of the *hypD* gene in denatured DNA. Primers 2 and 3 are compliments of each other. Primer 2 is named CECOR, and is 30 bases long. Primer 3 is named CECOF, and is also 30 bases long. They copy the *hypC* gene 3' to 5' and 5' to 3', respectively. In both of these primers, an *EcoRV* restriction site is contained within a part of the sequence that does not anneal to the *hypC* fragment in the second step of PCR. When the DNA polymerase copies the primer during elongation, it will match the complimentary bases to this segment of the primer. Thus, the sequences formed by primers 1 and 2 will contain an *EcoRV* site at one end, as will the sequences formed by primers 3 and 4 (Figure 2). The sizes of these fragments were found by use of gel electrophoresis. In this process, DNA fragments were placed in an agarose gel media, and an electric current was applied. The negatively charged DNA migrated towards the positive end

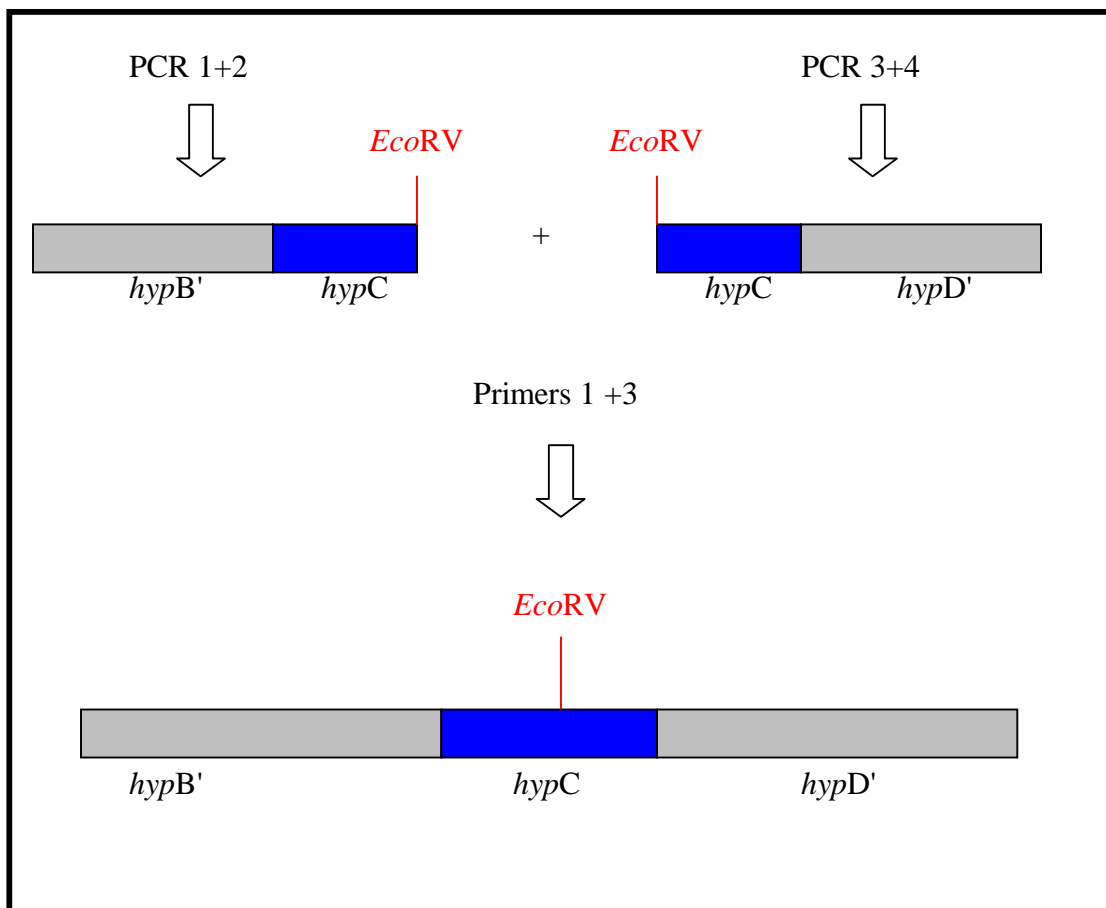
of the current. Molecular markers for fragment size were run in the gel at the same time, allowing the size of the unknown fragment to be accurately determined.

Figure 2. Primers Sequences Used During PCR and Products of First PCR. The *EcoRV* restriction site is shown in red in the sequences of primers 2 and 3. The designation of *hyp* B' or -D' represents that only a portion of the gene was copied in PCR.



In order to fully incorporate the restriction site into the *hypC* gene, PCR was conducted on the fragments generated from primers 1+2 and primers 3+4. Primers 1 and 3 were used during this second PCR in order to generate fragments that contained an *EcoRV* site within the gene (Figure 3). The size of this fragment was determined by gel electrophoresis.

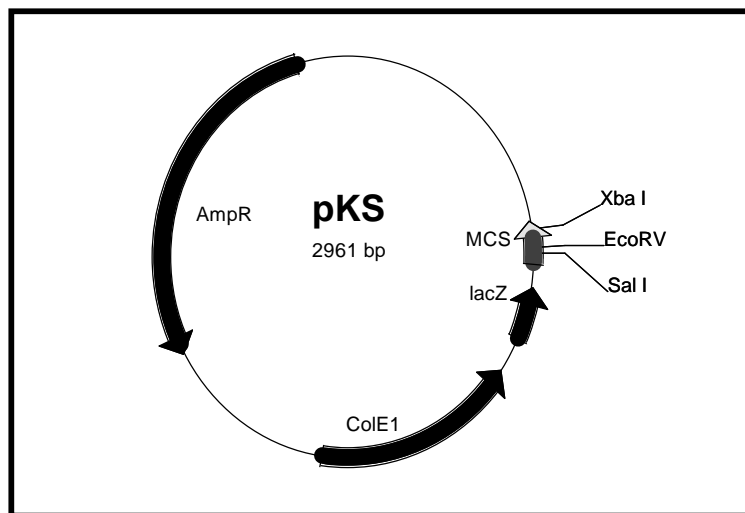
Figure 3: Creation of *EcoRV* Site Using Overlapping PCR.



Selection of a Plasmid Vector for the *hypC* Fragment. The pBluescript II KS (+/-) phagemid, or pKS, was chosen as the vector for the *hypC* fragment. A phagemid is a hybrid of a phage and a plasmid. A phage is a virus that can have its DNA integrated into the chromosome of a bacterium, while a plasmid is an extra chromosomal piece of DNA that can replicate independently of the chromosome. A cross between these two yields a phagemid, or a piece of DNA that can be cloned and expressed within the chromosome of the host cell (18). The pKS phagemid was specifically selected because of its convenience in cloning and in the detection of recombinant clones. This 2961 bp plasmid contains a multiple cloning site (MCS) where it can be cut using many different restriction enzymes. A DNA fragment can then be inserted into the cleaved MCS. Plasmids that have successfully incorporated the insert into their MCS are known as recombinant plasmids, and can be detected in two ways when using this plasmid. First, pKS contains a cassette for ampicillin resistance. If cells are plated on media that contain ampicillin, only cells resistant for this antibiotic should grow. The use of blue/ white complementation is the other method this plasmid provides for the detection of clones. pKS contains the *lacZ* gene upstream of the MCS. The promoter required to turn on this operon is located downstream of the MCS (Figure 4). If the MCS is interrupted with a fragment of DNA, the promoter does not reach the operon, and the *lacZ* gene is not activated. Plasmids are plated on media containing 5-bromo-4-chloro-3-indolyl-beta-D-galactoside, or X-Gal, and isopropylthio-beta-D-galactosidase, or IPTG, in order to determine the *lac* phenotype of the cells. Plasmids that have fully functional *lacZ* genes will be induced by IPTG

to produce beta-galactosidase, which hydrolyzes X-Gal. The products of this hydrolyzation form a blue precipitate on the media (19, 20). Plasmids that do not have functioning *lac* genes will not form this precipitate, and thus appear as white colonies on the media. Therefore, the use of this plasmid should yield ampicillin resistant white clones for those plasmids that have successfully incorporated the *hypC* fragment into the MCS.

Figure 4: pKS Phagemid Structure. The MCS, with the *Xba* I, *Sal* I, and *EcoRV* restriction sites is shown in gray. The ampicillin resistance cassette and *lacZ* gene are shown in black.



Construction of pRDD1. The *EcoRV* restriction site in the MCS was selected as the site for insertion of the *hypC* fragment. This site was chosen in order to inactivate it in the plasmid, therefore making the newly generated restriction site in the fragment the only *EcoRV* restriction site in recombinant plasmids. This new site can then be interrupted with a kanamycin resistance cassette to create mutants for the *hypC* gene.

The *EcoRV* site in the MCS was cut using the *EcoRV* restriction enzyme (Fisher). This enzyme recognized the nucleotide sequence 5' GATATC 3' and cleaved the bond between the first T and A. This yielded the following sequence: 5' GAT_ ATC 3'. The cut plasmid was treated with calf intestine phosphatase (Roche) in order to remove the phosphate from the ends of the plasmid. This prevented the plasmid from recombining once it has been cut, and allowed for integration of the *hypC* fragment at this cleaved site.

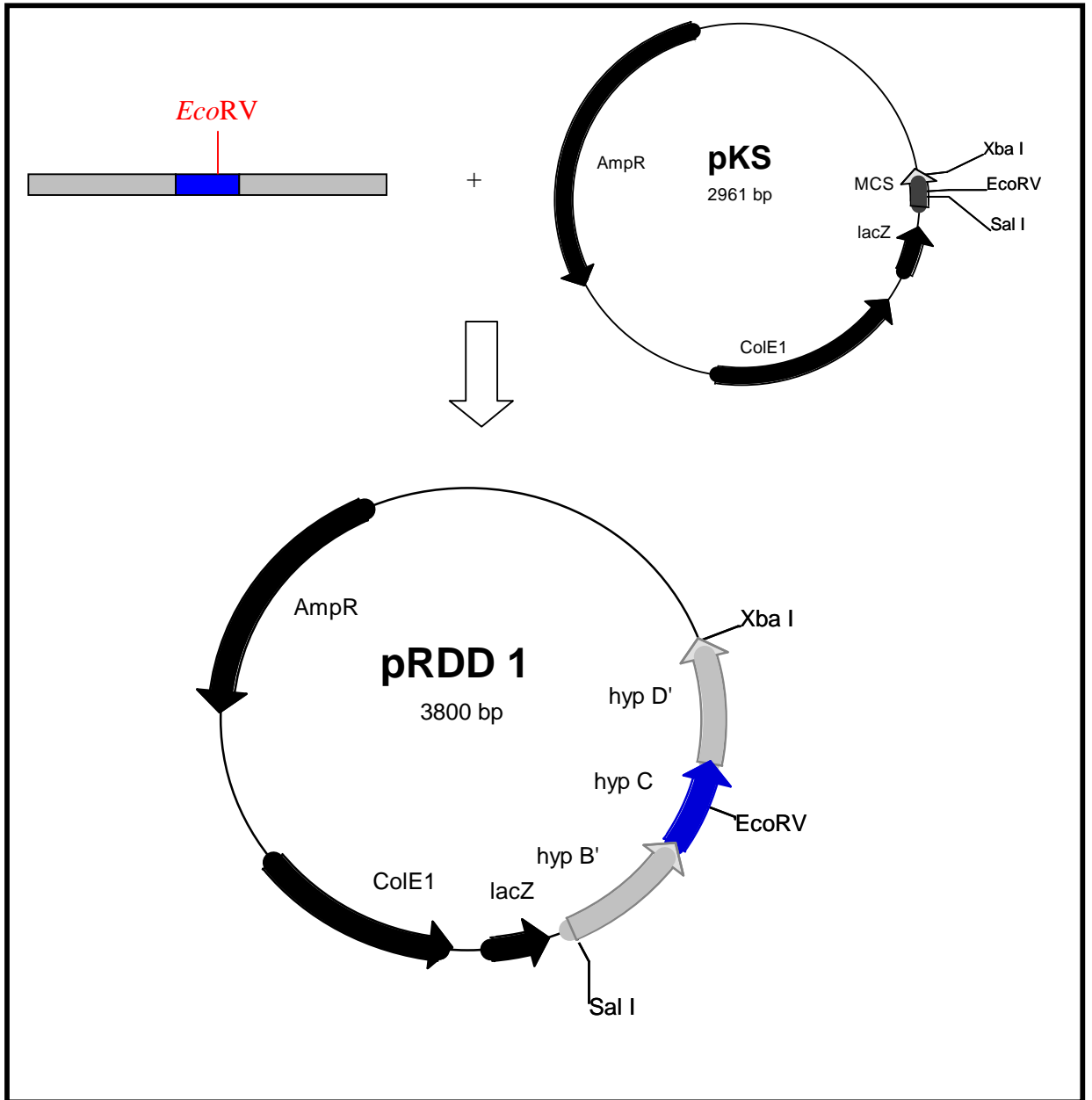
In order to incorporate it into the MCS site, the *hypC* fragment was first blunt ended by the use of T4 polymerase (Fisher). This enzyme filled protruding ends of the 5' strand of DNA with nucleotides and used exonucleases to create blunt ends from overhanging ends of 3' DNA. The blunt ended fragment was then treated with ATP as a source of phosphates. This enabled the insert to form bonds with the plasmid during ligation, as the phosphates had been removed from the plasmid blunt ends.

The insert and plasmid were ligated together using T4 DNA ligase (Invitrogen). This enzyme catalyzed the joining of the 5'-phosphate group of the insert DNA to the 3'-hydroxyl group of the plasmid DNA. The ligation mixture was

transformed into *E. coli* cells that had been treated with calcium chloride in order to make their cell walls more permeable to the vector - i.e. to make the cells complement. *E. coli* cells were plated on LB agar supplemented with ampicillin, X-Gal, and IPTG, and allowed to grow at 37 °C overnight. During this growth process, the DNA of the vector was incorporated into the genome of the bacteria. Therefore, all offspring of the cells contained the plasmid DNA. Recombinant clones were selected using blue/white complementation. White clones were selected as those that contained the insert successfully ligated with the plasmid.

In order to verify presence of the insert in the vector, plasmid DNA was removed from the white cells using QIAprep Miniprep Plasmid Kit for DNA. In this process, the cell was lysed and all of its contents, with the exception of DNA, degraded using enzymes. The DNA was filtered out and suspended in water. A sample of this was taken and cut using the restriction enzymes *Xba* I and *Sal* I, which have restriction sites located on either side of the *EcoRV* site in the MCS (Figure 4). The length of nucleotides between these sites is known to be 57 base pairs. If the *hypC* fragment was incorporated into the *EcoRV* site, the length of the fragment would be increased. The actual length of the fragment was determined by gel electrophoresis. Clones that were ampicillin resistant, white in appearance, and verified to have the *hyp* insert by electrophoresis were renamed pRDD1 (Figure 5).

Figure 5: Plasmid pRDD1. The *hypC* gene is shown in blue, the *EcoRV* site in red.

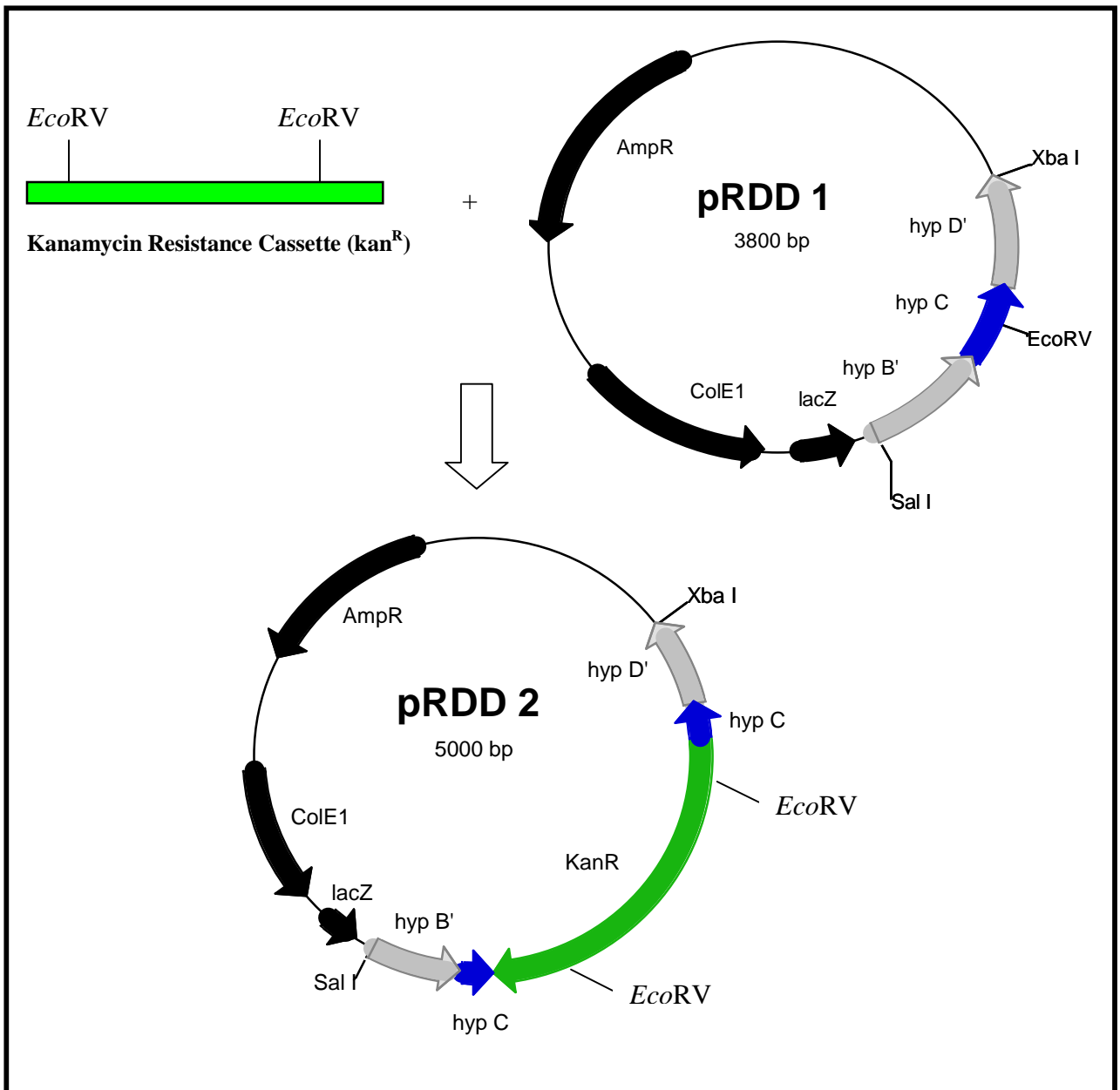


The Insertion of a Kanamycin Resistance Cassette into pRDD1. Plasmid pHP1 was digested with the restriction enzyme *EcoR1*, which released a 1.3 kilobase (kb) kanamycin resistance cassette (kan^R). The cassette was separated from pHP1 by use of gel electrophoresis, and extracted from the gel using the QIAquick Gel Extraction Kit (Qiagen). Here, the agarose gel was solubilized using a buffer solution. The kan^R was filtered out and blunt ended using T4 DNA Polymerase. It was then ligated to pRDD1 that had been digested with the restriction enzyme *EcoRV* and cut in the *hypC* gene. The ligated plasmid/ kan^R was then transformed into *E. coli* cells that had been treated with calcium chloride. The transformed cells were plated on LB plates supplemented with kanamycin and grown at 37 °C overnight. Only cells that had successfully integrated the kan^R were capable of growth on this media.

Verification of the presence of the kan^R in the cells was made using several different methods. Plasmid DNA was extracted from the cells, digested with *Xba I* and *Sal I*, and resultant fragment size determined by gel electrophoresis using the methods described above. The DNA was also digested with *EcoRV* as a means to ensure the presence of the insert. The kan^R contains two *EcoRV* restriction sites approximately 1 kb way from each other. These sites were the only ones of their kind available in the plasmid, as the *EcoRV* sites within the pKS plasmid and the *hyp* gene had both already been interrupted. The size of the fragments generated after this digestion was also determined using gel electrophoresis. The DNA was sequenced by the University of Georgia Molecular Genetics Instrumentation Facility as the final

method for verification of the presence of the kan^R . Plasmids verified to have the cassette were renamed pRDD2 (Figure 6).

Figure 6: Plasmid pRDD2. The kan^R is shown in green, the *hypC* gene in blue.



Transformation and Determination of Mutagenesis in *H. pylori*. *H. pylori* cells were transformed with pRDD2 using electroporation. Cells were grown on nonselective BA plates for three days. Plates were swabbed, and cells suspended in a washing solution of 15% glycerol and 9% sucrose. Cells were then centrifuged and suspended in the washing solution. This process was repeated a total of three times, and the final product suspended in 300 milliliters (mL) of washing solution. Three mL of pRDD2 DNA was then combined with 50 mL of the washed cells. An electropulser with current of 2.47 kilovolts was then applied to the cell. This instrument sends out short electric impulses that are thought to make membranes more permeable by disturbing the phospholipid bilayer. This disturbance allows the formation of pores in the membrane to occur without permanently damaging the cell. Molecules such as DNA can then enter the cell more easily (21).

After electroporation, the cell solution was plated on nonselective BA plates and allowed to grow for three days. During this time, the pKS phagemid inserted the mutated *hyp* gene fragment into the chromosome of the *H. pylori* cells. After three days, cells were then streaked on BA plates containing kanamycin and allowed to grow for two more days. Four clones were selected from these plates, and the chromosomal DNA extracted using the methods described above. The *hypC* DNA was then amplified by use of PCR and the primers *hypC-R2* and *hypC-F*. Gel electrophoresis was then conducted to determine the size of this gene in comparison to wild type DNA and the positive control of pRDD2.

Hydrogenase Enzyme Assays. Hydrogen uptake was determined amperometrically as described by Wang *et al* (22). Cells were grown on nonselective BA plates for two days in a CampyPak plus environment. They were then harvested and washed in 3 ml of a 50 molar solution of HEPES-sodium hydroxide (Noah) with a pH of 7.5. The wash process was repeated twice using the same buffer, and the cells were resuspended in approximately 1 ml of the solution after the final wash. Optical density (O.D.) readings were then made using a Beckman DU640B spectrophotometer. This instrument transmits light at a certain wavelength through a liquid sample in order to detect the percent of light absorbed or scattered by the sample. The more light scattered by the sample, the higher the number of cells in the solution. The number of cells in the HEPES-NaOH solution was then estimated by determining the amount of light scattered at 600 nanometers.

After determining the O.D., approximately 2.5×10^9 cells were injected into an anaerobic chamber containing 37.7 nanomoles (nmoles) of hydrogen. A platinum cathode was located in the middle of this chamber, and a silver anode was located on the side. The ability of a current to flow between these two electrodes when a potential of + 60 volts was applied was dependent on the presence of hydrogen within the chamber. Thus, the rates of hydrogen uptake could be determined for each cell solution by measuring how long the current was conducted between the two electrodes. The measurement of current between two electrodes to determine the end point of a reaction is known as amperometric measuring.

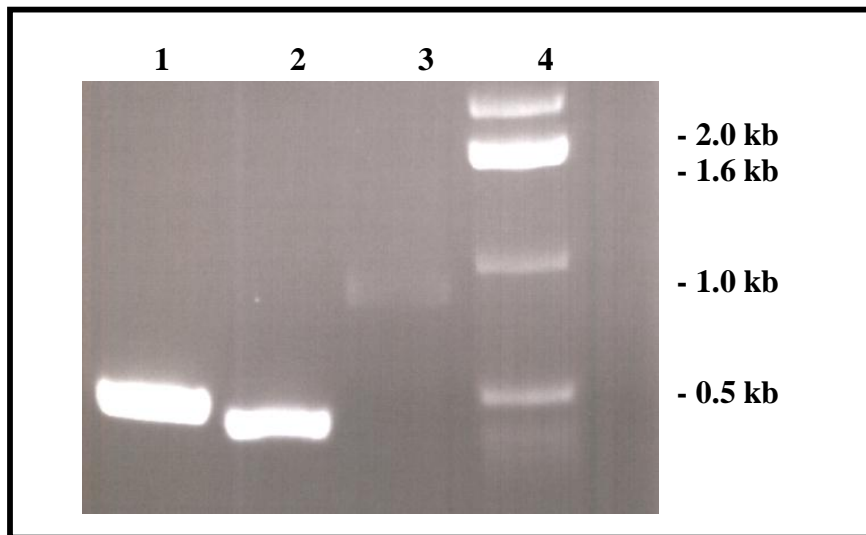
Urease Enzyme Assays. Quantitative urease assays were determined using the phenol-hypochlorite assay described by Weatherburn (23). This assay was conducted twice in order to verify the results to be reproducible. *H. pylori hypC* mutant cells were harvested from BA plus kanamycin plates after a two day incubation period. The cells were washed with 1 mL of a 50 molar HEPES-NaOH solution. The cells were then broken by sonication, a process that uses ultrasound vibrations to destroy the cell membrane. Protein was extracted from the cell, and added to a mixture of the HEPES buffer and a 25 molar urease buffer solution. This solution was incubated in a 37 °C water bath for 20 minutes to allow the urease in the buffer to convert urea into ammonia. After precisely 20 minutes, 1.5 mL of a phenol-nitroprusside sodium solution was mixed into the solution. 1.5 ml of a sodium chloroxide - NaOH solution was immediately added after mixing. The solution was then allowed to incubate for an additional 30 minutes in the same conditions.

Optical density readings were then made using the spectrophotometer. By comparing the absorbance of an unknown concentration to that of known sample concentrations, an unknown concentration can be determined. For this experiment, a wavelength of 625 nanometers was used, as it is the established wavelength for maximum absorbance to occur (21). Absorbance was found for each sample and compared to the absorbance of wild type 43504, which was used as a control for this experiment. Urease activity was found by determining the nmoles of urea hydrolyzed per minute per microgram of protein in the solution.

Results

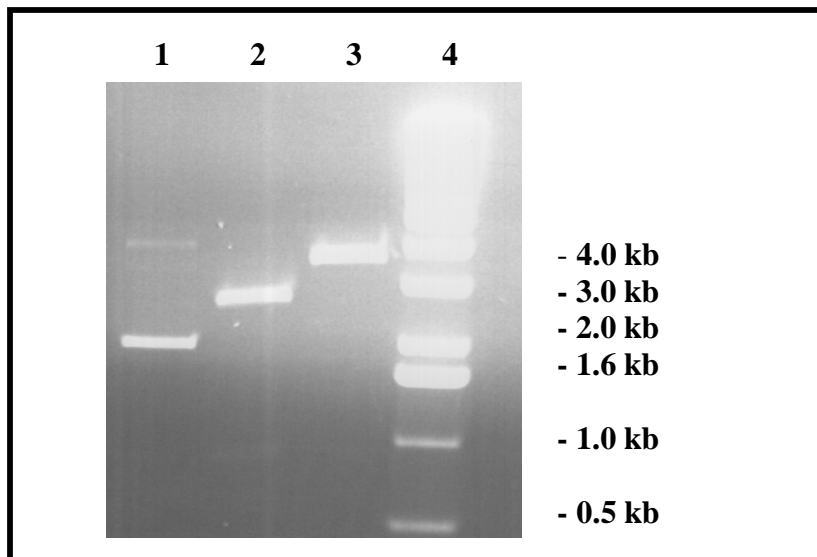
Determination of Size of PCR Fragment by Gel Electrophoresis. The two gene products generated during PCR using primers 1,2,3, and 4 were determined to be approximately 435 and 485 base pairs (bp) in length. The gene fragment produced by overlapping PCR using primers 1 and 3 was found to be approximately 900 bp in length. The results of both of these gels can be seen in Figure 7.

Figure 7: Gel Electrophoresis Results for PCR. Lane 1 shows the 485 bp PCR fragment created using primers 1+2, while lane 2 shows the 435 bp fragment generated using primers 3+4. Lane 3 shows the 900 bp fragment created during overlapping PCR. Lane 4 contains the DNA standard, with known fragment sizes indicated in kilobases (kb) on the right.



Verification of PCR Fragment in Plasmid pRDD1 by Gel Electrophoresis. The size of the pKS phagemid is known to be 2.9 kb. The insertion of the *hyp* gene fragment increased the size of the phagemid by 900 bp, making it approximately 3.8 kb in length. This plasmid was renamed pRDD1. When DNA was cut with the restriction enzymes *Xba* I and *Sal* I, the insert was essentially removed from plasmid pRDD1. This yielded a 900 bp fragment and a 2.9 kb fragment, as corresponds to the original size of the fragment and plasmid before ligation. pRDD1 DNA was also cut with the restriction enzyme *EcoRV* to verify the presence of the newly created restriction site in the *hypC* gene. This cut resulted in a single piece of linearized DNA that was 3.8 kb in length. The results of the gel in figure 8 verify the presence of the *hyp* insert with an *EcoRV* restriction site in plasmid pRDD1.

Figure 8: Gel Results of Plasmid pRDD1. Lane 1 shows the linearized piece of DNA obtained when cut with *EcoRV*. Lane 2 shows the 900 bp fragment and the 2.9 kb fragment generated by cutting with restriction enzymes, while lane 3 shows the positive control of uncut pRDD1. Lane 4 contains the DNA standards.

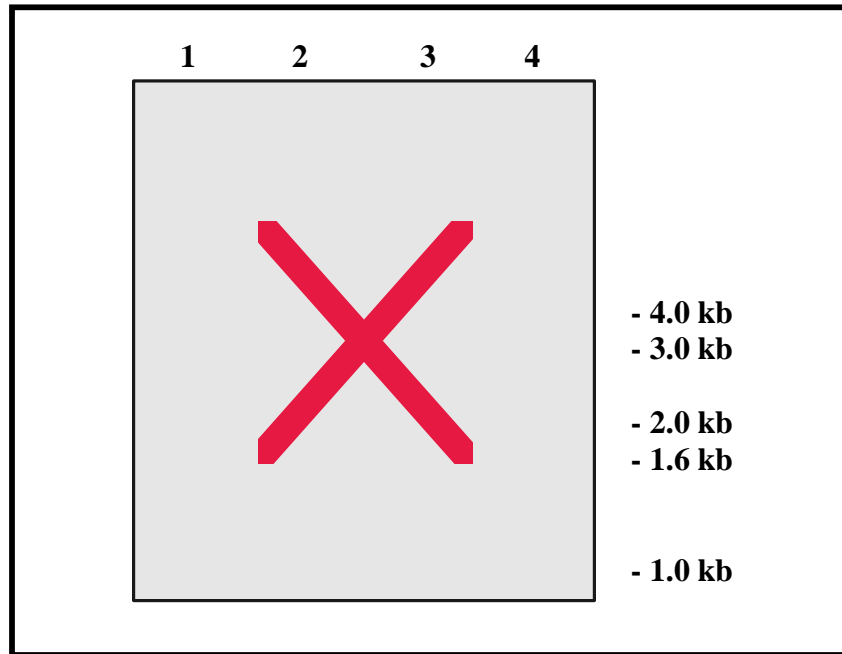


Verification of kan^R in Plasmid pRDD2 by Gel Electrophoresis and Genetic

Sequencing. The length of plasmid pRDD1 increased by 1.3 kb when ligated with the kan^R, yielding a 4.3 kb product that was renamed pRDD2. Plasmid DNA was extracted and digested with *Xba* I and *Sal* I to confirm the incorporation of the kan^R within *hypC*. This resulted in two fragments of approximately 1.3 kb and 2.9 kb, as corresponds to the original size of the plasmid and kan^R. When ligated together, the *EcoRV* site in the *hypC* was disrupted by the kan^R. Therefore, the only *EcoRV* sites in pRDD2 should be those found within the kan^R (see Figure 6). When digested with *EcoRV*, a 4.0 kb fragment and a 1.0 kb fragment was generated. These results verify the successful incorporation of the kan^R into the *hypC* gene, and can be seen in Figure 9.

The presence of the kan^R was also verified by sequencing by the Molecular Genetics Instrumentation Facility (MGIF) at the University of Georgia. The kan^R is shown to be in the expected site and divergently transcribed, or transcribed in the opposite direction, with respect to *hypC* (see Figure 6). The resultant gene sequence is shown in Appendix C.

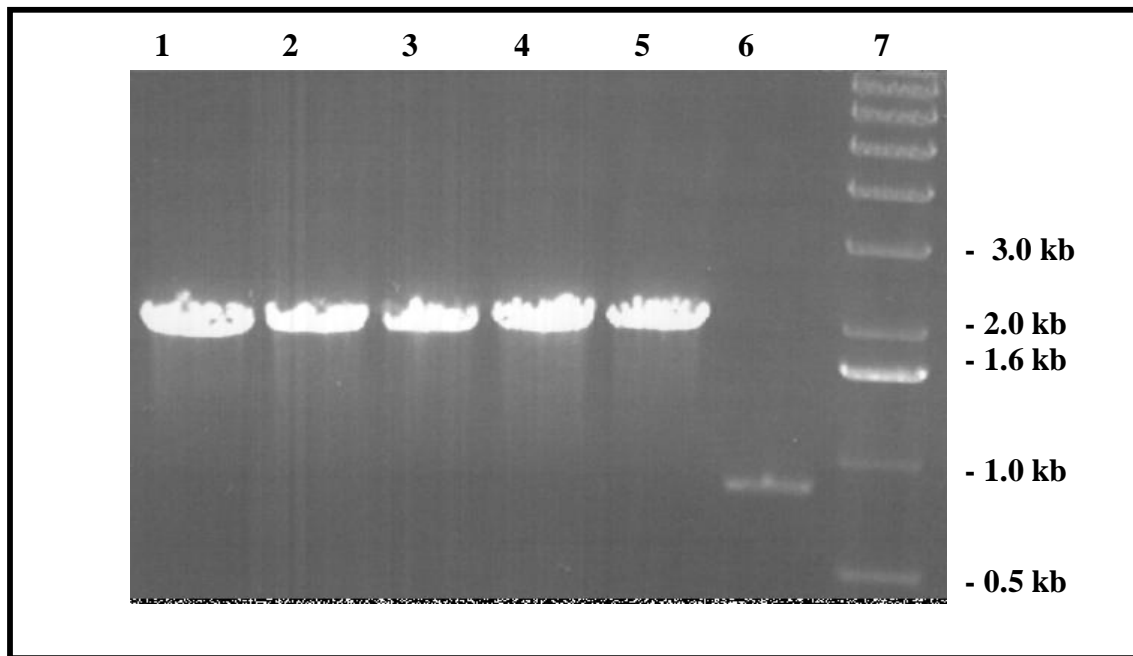
Figure 9: Gel Results for Plasmid pRDD2. Lane 1 represents uncut pRDD2, while lane 2 shows the fragments generated by digestion with *Xba* I and *Sal* I. Lane 3 shows the fragments generated by digestion with *EcoRV*, and lane 4 contains the DNA standard.



Verification of Mutagenesis of *H. pylori*. After the electroporation process, 28 colonies were observed when cells were incubated on BA + kan plates. Eight of these colonies were selected and restreaked onto BA + kan plates and allowed to grow for two days. Of the colonies observed after the incubation period, the four colonies that appeared to have the best growth were transferred to fresh BA + kan plates and labeled C1, C2, C3, and C4. Chromosomal DNA preps were conducted for all, and

the resultant DNA amplified using PCR. Agarose gel results confirmed the presence of the mutated *hypC* fragment in the chromosome by showing an increased in size in C1, C2, C3, and C4 when compared to wild type 43504 (Figure 10). This increase corresponds to the presence of the kan^R in the clones.

Figure 10: Gel Results Confirming *H. pylori* Mutagenesis. Lanes 1-4 show the clones C1, C2, C3, and C4. Lane 5 shows the positive control of pRDD2, while lane 6 shows wild type 43504. Lane 7 contains the DNA standard.



Hydrogenase Assay. As shown in Table 1, all mutants studied were shown to be affected for hydrogenase activity when tested. This was expected, as the *hypC* gene has previously been shown to play an integral role in hydrogenase maturation and function. These results correspond to the findings of the Olson experiments, and to the proposed role given to *hypC* when the *H. pylori* genome was sequenced (10, 17).

Table 1. Hydrogenase Activity of *hypC* mutants. Activity is expressed in nmoles of H₂ oxidized per minute per 10⁻⁸ cells. The values for this assay are reported as the mean ± standard deviation.

Strain	Hydrogenase
43504	0.8 ± 0.1
C1	< 0.001
C2	< 0.001
C3	< 0.001
C4	< 0.001

Urease Assays. As seen in Table 2, all mutants were shown to be affected for urease activity as well. The results show a marked difference in enzyme activity for the mutants when compared to the wild type activity of 19243 ± 205 nmoles of urea hydrolyzed per minute per microgram of protein in the solution.

Table 2. Urease Activity of *hypC* mutants. Activity is expressed in nmoles of urea hydrolyzed $\text{min}^{-1} \text{mg}^{-1}$ protein. The value for this assay is reported as the mean \pm standard deviation.

Strain	Urease
43504	19243 ± 205
C1	3241 ± 258
C2	2565 ± 159
C3	206 ± 18
C4	251 ± 69

Discussion

The results of this study suggest that the proposed goals of this project were met. A functioning *EcoRV* restriction site was created in the *hypC* gene, as verified by gel electrophoresis. Mutants for *hypC* in *H. pylori* were generated by the insertion of a kanamycin resistance cassette into this restriction site, as confirmed by both gel electrophoresis and genomic sequencing. Assays were conducted to determine the affects of mutation on hydrogenase and urease activity.

The hydrogenase accessory protein HypC was found to be required for full urease activity in *H. pylori*. All mutants observed were shown to hydrolyze smaller amounts of urea when compared to wild type 43504. This assay was repeated twice to account for experimental error. Results were found to be comparable for both runs, verifying that these assays are reproducible. The urease activity observed for clones C3 and C4 were especially interesting in that they exhibit 100 fold less activity than the wild type. These mutants also appear to be more affected for enzyme activity when compared to mutants C1 and C2. This difference could be the result of the expression of different phenotypes by the mutants.

In order to verify that it is the absence of the HypC accessory protein that is causing the lower rates of urease activity, mutants can be grown on media supplemented with nickel. Nickel supplementation led to a 40% restoration of the activity of this enzyme in *hypA* and *hypB* mutants during the Olson experiments, and is expected to restore activity in *hypC* mutants as well. Urease activity is also

expected to be restored if an uninterrupted copy of the *hypC* gene is inserted into the mutant. Due to time restraints and equipment limitations, these experiments were unable to be conducted at this institution. This paper reports that these experiments are currently being conducted in the University of Georgia Microbiology Department, Athens, GA.

The results of this project compliment the Olson project because they reveal the affects of a previously unstudied *hyp* mutant on urease activity. The role of the accessory proteins encoded by every *hyp* gene has now been determined. The proteins encoded for by *hypA*, *hypB* and *hypC* genes were all found to affect urease activity in addition to hydrogenase activity. These results of both of these projects are unique in that before the Olson findings were reported, the accessory proteins encoded for one nickel enzyme had not been shown to affect another nickel enzyme (10).

The results of this experiment and the Olson experiment were unexpected because there are many differences between the hydrogenase and urease enzymes. These enzymes are similar in that they are both metalloenzymes, and therefore require nickel to be fully active. However, it has been shown that there is little homology between their primary and secondary structures, and that they play different roles in cellular functioning (10). Hydrogenase degrades molecular hydrogen to provide hydrogen ions and electrons to be used during metabolism, whereas urease breaks down urea to buffer the cell against the acidic environment of the stomach during colonization. Nickel binding also differs in that urease uses

nitrogen and oxygen to ligate with two nickel ions, while hydrogenase uses sulfur to bind only one nickel ion (10). Despite these differences, the accessory proteins required for full functioning of both enzymes were found to overlap.

These results were also unexpected because previous research had not revealed any *hyp* accessory proteins as factors that increased urease function within *H. pylori*. McGee *et al* identified several factors for urease activity by determining activity levels when all of the *ure* genes were expressed in *E. coli*. Although this gene cluster seemingly contains all of the factors required for full enzyme function, urease expression levels were found to be lower in these cells when compared to urease expression rates in *H. pylori* (24). The diminished ability of *hypA*, *hypB*, and *hypC* mutants to express full urease activity implicates these proteins as the missing factors required for fully functioning urease enzymes.

This project reveals the role of the hydrogenase accessory protein HypC in the functioning of the enzyme urease. Progress in understanding the roles of different molecules involved in colonization and persistence could lead to the development of more effective therapies or vaccines against *H. pylori*. Hydrogenase has already been identified as a possible site for inhibition of this bacterium because it contains unique enzymes and structural domains not found in humans (25). It has been proposed that vaccines could be developed to specifically target the urease enzyme as well (8). Findings by studies such as this one could lead to a better understanding of the regulation of urease in *H. pylori*. This may lead to novel anti-urease therapies

against *H. pylori* and other urease-producing pathogens, which could help decrease the incident of peptic ulcers and gastric cancers worldwide.

Appendix A: Genomic Sequence for Kanamycin Resistance

The DNA sequence for this resistance gene is 816 nucleotides long. The numbers on the left side of the column correspond with the number of the first nucleotide in that row. For example, the number 61 tells you the first letter 'g' of row 2 is the 61st nucleotide in this sequence. Columns are broken into groups of ten nucleotides so as to make counting easier.

```
1  atgagccata ttcaacggga aacgtcttgc tcgaggccgc gattaaattc caacatggat
61  gctgatttat atgggtataa atgggctcgc gataatgtcg ggcaatcagg tgcgacaatc
121  tatcgattgt atgggaagcc cgatgcgcca gagttgtttc tgaaacatgg caaaggtagc
181  gttgccaatg atgttacaga tgagatggtc aggctaaact ggctgacgga atttatgcct
241  cttccgacca tcaagcattt tatccgtact cctgatgatg catggttact caccactgcg
301  atcccagggg aaacagcatt ccaggtatta gaagaatata ctgattcagg tgaaaatatt
361  gttgatgcgc tggcagtgtt cctgcgccgg ttgcattcga ttctgtttg taattgtcct
421  tttaacggcg atcgcgtatt tcgtctcgc cagggcgaat cacgaatgaa taacggtttg
481  gttggtgcga gtgattttga tgacgagcgt aatggctggc ctgattgaaca agtctggaaa
541  gaaatgcata agcttttgcc attctcaccg gattcagtcg tcaactcatgg tgatttctca
601  cttgataacc ttatTTTTga cgaggggaaa ttaataggtt gtattgatgt tggacgagtc
661  ggaatcgcag accgatacca ggatcttgcc atcctatgga actgcctcgg tgagttttct
721  ccttcattac agaaacggct ttttcaaaaa tatggtattg ataatcctga tatgaataaa
781  ttgcagtttc acttgatgct cgatgagttt ttctga
```

Appendix B: Genomic Sequence of the *hypB*, -C, and -D.

The *hypD* gene is 1112 nucleotides in length, and is shown in gray from 5804 – 6916. The *hypC* gene is from 6922 - 7155, and is represented by blue. The *hypB* gene is 728 nucleotides long, and is also represented in gray from 7155-7883.

```
5761 gcaaatagtg agcagtctag gggcttagag caatdddcaa aactcaaacg
cgcttgtaac
5821 gataatacgc cgcgcacgcc cccctcagagc tgaccatgca actgccgatc
gggtdtttgtag
5881 ggggtgcaagt tgtagcgaat aacgagcagt ctaggggctt agcgatgcct
tttaaaatct
5941 ccccgcaact gcatgctttg ttttctttag aggttttgtag gcttaaatat
tctttaaaga
6001 cttctttcagc gtcataagaa gcgaacgctt ctttgagttt gagagcggat
cgtttgatat
6061 tccccaaacc tctccattca aaatdddccc taacttccat gcaagcattg
actaactctt
6121 gcgctttcac attcccctca aaactcaccg ctcttttgta ttggatttct
agcttggtt
6181 ctttgtttag ggcttgtttg ataagcatca gcacgctttc taatatatcc
accggctcaa
6241 aaccgctcac gataatgggg attdtaaaagc gatccactaa aggagcatag
attdgagcgc
6301 cgctgatcac gctcacatgg ctaggggcta aaagggcgctt aatctggcat
gctggatctt
6361 ttaaaatcgc gctcacgctg ggaggcacta gaatgtgggtt aatgtggaaa
aaaaggttat
6421 taattdtttc ttdtttggtg ctccataaaa cgctagcgcct catggggcgctt
gtggtdttcaa
6481 aaccgatcgc aatataaatg acttdtttttag tagggtdtttc ttdtagcgcct
tctaaagctt
6541 gcatgggcca atacaaaaag cgtgcatcca accccttdtc tctggcttgt
atcaaactcc
6601 catagctccc ggggactctc atcatatccc cttaaactcaa aacaatgcta
tcttdgatag
6661 tagcgagttc ataagcttca tcaaggcgcg ctcttggtcat cacgcatacc
gggcagcccg
6721 gcccatgcac aaactctaaa ttgttaggca tcaaactctaa aagcccgtat
tctatgatag
6781 aatgcgtatg cccctcgcac acttccatga tgactaattt ttdttcaagt
ttgaaagcga
6841 gtdtdtttgat tgcattagag agcgctaaaa gggtdcgcct gtctctaaag
ggcgaaatga
6901 ggtgatcaac gctcattggt attattdcgt ttcgtdcatt ctggcgatca
tdtcttgata
6961 aagctcaatg gattctaggg ctdttdtttc atcaatcttg ctcatcacat
agccgatgtg
7021 caacagcaca taatcgccca ctdtaacgga ctgcgccatt aaatccaagc
tcgctctct
```

7081 ttgaacgccc aaagttcca aaagcaccac attatcctta atggctatga
ctttagaggg
7141 gatcgctaaa cacatataaaa cgaatgcgtg gactggtaat tttcacgctt
tttttctaga
7201 aggaaatddd taaaatcttc caaactttta gggctcttag agctcattaa
aaaaataggg
7261 gcttcaggct ttaatdddttg catgtcttct ttgacttgag aaaccctgaa
attaaacacc
7321 tcaaccatat ccgctttact gataatcacc gcatccgctc acatgaacat
cgtaggggat
7381 tttagcacct tatcatcgcc ctctggaacg gagagtaaaa cgatattcat
cgccgctcct
7441 agattatagc ttgaggggca aaccaaattc cccacgtttt caatgattaa
aaaatcgctt
7501 ttttctaacy ctccctcacc ttttaataaa tcaaacgccc cttcaatcat
gctcgcttcc
7561 aaatggcatg cttcgccggg ggtgatctgg tgcgcactca cgcctttttt
acgcaatctg
7621 tccgcatctc tgttggtttg caaatcgccc tctaccacgc aaaacttaaa
gtctttaaaa
7681 tccgctagat tttctagcat cgtggtttta ccgctaccgg gagaactcat
gaaattcaac
7741 acatacagcc cttcttttaa atagcgttct ttcatttcag cggctttaat
gctgcttctta
7801 ctcaaaatct tttctacgat tttgacatct tttttactca aattaggggt
atdddgtaaa
7861 gattcttctc gttgttcgct catgttgctt ctttctttta aaatdddctg
atcgtagcgc

Appendix C: Genomic Sequence Verification of Kan^R

Two sequences of genomic pRDD2 were received from the Molecular Genetics Instrumentation Facility. Sequence A shows kan^R inserted in *hypC* and the *hypD* gene. Sequence B shows the insert, *hypC*, and the *hypB* gene. These sequences show the kanamycin resistance cassette was incorporated into the *EcoRV* site in the *hypC* gene. The kanamycin resistance cassette is shown to be inserted in the opposite direction of the *hyp* genes.

hypD' and *hypB*' are shown in gray, while the *hypC* gene is shown in blue. The kan^R is shown in orange. The letter 'N' is used to designate those nucleotides that could not be accurately determined during the sequencing process.

Sequence A: *hypD*', *hypC*, and kan^R

```
CTTGTATCAAACCTCCCATAGCTCCCGGGGACTTTCATCATATCCCCTA
AACTCAAAACAATGCTTATCTTTGATAGTGGCTAACCCATAAGCGTCA
TCAAGGCGCGCTCTTGGCATCACCGCATAACCGGGCAGCCCGGCCATG
CACAAAACCTCCTAAATTGTTAGGCATCAAATCCAAAAGCCCGTATTTT
ATGATCGAATGCGTATGCCCTCCGCACACTTCCATGATGACTAATTTT
TTTTCAAGTTTGGAAAGCGAGTTTTTTTGATTGCATTAGAGAGCGCTAAA
AGGGTTCGCTTGTCTCTAAAGGGCGAAATGAGATGATCAACGCTCAT
TGTTATTGCGTTCGTTTCAATTTTGGCGATCATTCTTGATAAAGCTCAA
TGGATTCTAGGGCTTCTTTTCATCAATCTTCAACTTGCTCATCACATA
GCCGATGTGGAGCAGCACGATAATTCCTATGAAGCGCCATATTTAAA
AAGCTACCAAGACGATAAATGCGTTCGGAAAGTTAAACTGCGAAAAAT
TGGAACCGGTACGCTTATATAGAAGATCGCCGTATGTGTAAGGATTC
AGGGGGCAAGGCATAGGCAGCGCGCTTATCAATATACTSTSGAATGG
GCAAAGCATAAAAACCTTGCATGGACTAATGAAACCCNGAANATAACC
TTATAGCNTGGAAATNCTATCAAAAANTGGGGGNTTCCAAAATCGGCT
NCCNCCNAA
```

Sequence B: *hypB'*, *hypC*, and *kan^R*

CGATGAATATCGTTTTACTCTCCGTCCCAGAGGGCGATGATAAGGTGCTA
AAAATACCCACGATGTTTCATGTGTGCGGATGCGGTAATTATCAGTAAAG
CGGATATGATTGAAGTGTTAATTTTCAGGGTTTCTCAAGTCAAAGAAGAC
ATGCAAAAATTAAAGCCTGAAGCGCCTATTTTTTTAATGAGTTCCAAAGA
TCCTAAAAGTTTGGAAGATTTTAAAACTTCCTTTTAGAAAAAAGCGTG
AAAATTACCGTCCACGCATTCGTTTTAATGTGTCTAGCGATCCCCTCTAAA
GTCTTAGCCATTAACGTAATGTGGCGCTCTTAGAGATTTGGGCGTTCAA
GAGAGGCCGAGCTTGGATTTAATGGGCGAGTCCGTTAAAGTGGGCGATAA
TTCAGACATCTAAATCTAGGTACTAAAACAATTCATCCAGTAAAATATAA
TATTTTATTTTATTTTCTCCCAATCAGGCTTGACCCAGTAAGTCAAAAAAT
AGCTCGACATACTGTTCTTCCCCGATATCCTCCTCGACCGACGCAGAAGG
CAATGTCATACCCTTGTCGGCCCTGCCGCTTCTCCAGATCAATAAAGCCC
TTNCTTTGCCATCTTTCCAAAAATNTGCTNTCCCCAGNCGCCGNGGGAAA
GACAATTCNNTCGGGNTTCCGNTTNAAAAA

Acknowledgements

1. Dr. Stephane Benoit, post-doc at the University of Georgia, for guidance and support throughout this project.
2. Dr. Robert J. Maier, laboratory mentor at the University of Georgia, for guidance and support throughout this project.
3. National Science Foundation, for the funding that made this project possible.
4. Provost Student Research Award for support through grant funding.
5. University of Georgia Research for Undergraduates in Prokaryotes (REU) Program Staff and Participants for support throughout this project.
6. Departmental Honors Committee Members for their time and support in the writing of this thesis.

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Construction and Characterization of *hypC* Mutants in
Helicobacter pylori

by
Rachael Davis

Project Director: Dr. Henry Spratt
Examination Date: November 14, 2002

Rebekah P. Bell
Dr. J. Hill Craddock
Dr. Edward Rozema

Examining Committee Signatures:

Project Director

Department Examiner

Department Examiner

Liaison, Departmental Honors Committee

Chairperson, University Departmental Honors Committee