The Role of Polyphosphate Kinase in Long Term Survival of *Helicobacter pylori*.

by

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ABSTRACT

Polyphosphate kinase may have a role in regulating the physiology of cells living in less than optimal conditions such as those facing bacteria in stationary and death phases. Experimental studies were performed to assess the role of polyphosphate kinase on the long term survival of *Helicobacter pylori.* I created isogenic mutant strains deficient in the production of polyphosphate kinase by targeted insertional mutagenesis using a kanamycin resistance cassette and collected strains from other laboratories.

Bacteria were grown to stationary phase, and then plated periodically to measure the decline in culturability. My mutants showed a decrease in colony forming unit survival after 10 days compared with their parent strain. However, a second isogenic pair constructed with a chloramphenicol resistance cassette did not show a decrease. My results are therefore equivocal.

Thesis Supervisor: David Schauer
Title: Associate Professor of Toxicology

Introduction

Polyphosphate and long term survival

Polyphosphate (PP) is a long chain polymer of phosphates that has been detected in every natural living cell (Akiyama, Crooke, and Kornberg 1992; Bode et. al., 1993). A few enzymes are known to process PP. Polyphosphate kinase (PPK) catalyses the reversible polymerization of PP from adenosine triphosphate (ATP). The physiological role of PP is not known. Because levels of PP change dramatically as cultures reach stationary and death phase, it has been suggested that PP plays a role in long term survival (Ault-Riche et.al., 1998; Kim et.al., 1998).

Although PP can drive the production of ATP, energy calculations show that it can not serve as a long term energy storage media because it could only power a cell for a matter of seconds. It is much more likely to serve a regulatory purpose (Blum et.al., 1997; Kumble, Ahn, and Kornberg 1996). PP concentration versus growth phase profiles have been measured for a variety of species. Some bacterial species have high levels of PP just before the end of the exponential growth period and very low levels after a short time in stationary phase. Other bacterial species only begin to accumulate PP after reaching stationary phase. Because there is considerable variation in the dynamics of PP accumulation among bacterial species the gene regulation framework is likely not well conserved.

The Viable But Not Cultured (VBNC) hypothesis

When faced with adverse conditions some gram negative bacteria, such as many *Vibrio* species, can enter a physiological state in which they do not grow on conventional media which normally supports growth(Barer 1997; Dixon 1998; Whitesides and Oliver 1997; Byrd, Xu, and Colwell 1991). However, the bacterial cells remain viable as measured by such techniques as differential single stranded versus double stranded DNA staining, and detection of active electron transport systems by the reduction of a colorless substrate, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT), to form a visible precipitate. Cultures with no cells capable of forming colonies on standard media can in some cases cause infection and disease in susceptible experimental animals, allowing recovery of normally culturable bacteria. (Cellini et.al., 1994[1])

There are no general rules concerning nutrient concentration, temperature, and osmotic stress which apply to the recovery of all species. In some cases, the optimal conditions for induction of a VBNC state and subsequent recovery to fully culturable cells have been discovered (Whitesides and Oliver 1997). The VBNC hypothesis is not universally accepted (Bogosian, Morris, and O'Neil 1998; Kusters et.al., 1997).

Helicobacter pylori

Helicobacter pylori is a gram negative microaerobic spiral shaped bacterium which undergoes a morphological change to a spherical coccoid form under such stresses as nutrient deprivation and sub-lethal antimicrobial insult (Bode, Mauch, and Malfertheiner 1993; Cellini et.al., 1994[2]; Benaissa et.al., 1996). It is the etiologic agent of peptic ulcer disease. Life-long infection is linked with an elevated risk of cancer in the stomach. *H. pylori* has been found in gastric biopsies, feces, and dental plaque of humans (Nguyen et.al., 1993). Although humans mount an immune response to *H. pylori*, the infection is usually not cleared, resulting in a life-long chronic infection. Few of those infected show any clinical signs of gastritis, despite clear evidence by histology.

In the developing nations, most people are infected before age twenty and the direct fecal-oral route of transmission is generally considered to be most important. The rate of infection in adults in the developed nations is about 1 percent per year. This is consistent with both the direct oral-oral route and the indirect via surface waters route of transmission (Lee, Fox, and Hazell 1993; Chuanfu et.al., 1996). Epidemiologic studies in Peru suggest that drinking municipal water, instead of drinking private well water, increases the risk for *H. pylori* infection.

Transmission of *H. pylori* via environmental waters has not been clearly demonstrated. Efforts to culture the organism from the environment have failed. However, PCR studies have shown the presence of

H. pylori DNA at locations which if the organism were to be cultured, a threat to public health would be clearly recognized (Hulten et.al., 1996; Hulten et.al., 1998). While a VBNC form of *H. pylori* is consistent with the available data on human infection patterns, the evidence for the existence of *H. pylori* in a VBNC state is not as robust as that for other organisms (Cellini et.al., 1994[1]; Eaton et.al., 1995; Cellini 1996; Eaton et.al., 1996; Wang et.al., 1997). The debate is further clouded by the spiral to coccoid morphological transformation which may or may not have any relevance to a death resistant or VBNC state.

My studies seek an understanding of the parameters which govern the loss of culturability. Such understanding is needed to design experiments to more definitively assess the possible existence of a VBNC state in *H. pylori*. The primary factor under investigation is the presence or absence of a functional PPK gene.

Preliminary survival studies were performed with the ATCC type strain and an isogenic mutant in which the PPK gene was inactivated by insertion of a kanamycin resistance cassette (aphA-3). In an attempt to control for any effect on survival of the antibiotic resistance marker, a second pair of *H. pylori* strains consisting of N6 and its isogenic urease (ureB^aphA-3) knockout were included (Ferrero et all, 1992). Each of the kanamycin resistant mutants was significantly impaired in long term survival. The relative difference between the two pairs was small. Urease catalyzes the hydrolysis of urea to form ammonia and carbon dioxide with an accompanying rise in pH. In the growth and storage conditions used, virtually no urea was present. In the absence of urea, urease is expected to play no biochemical role, although a role in membrane structure can not be ruled out. Therefore, the kanamycin resistance cassette may be responsible for most or all of the reduced long term survival of those mutants. I also use a PPK deletion mutant with a chloramphenicol marker, courtesy of Timothy McDaniels. Because a set of isogenic mutants in a single strain background with the proper controls was not assembled my results are equivocal.

Materials and Methods

Culture Conditions

E. coli DH5-α was grown in LB or on LBA at 37°C.
The *H. pylori* strains were grown out of permanent stock on Tryptic Soy Agar (TSA) supplemented with 5% defibronated sheep blood in a 5% $C0_2$ incubator at 37°C. A single colony of each strain was subcultured on a fresh plate. In the first two survival studies, *H. pylori* strains were grown in a single 24 hour passage in the optimal broth (OB) of Andersen et.al. [ref] modified by using defibronated sheep blood instead of citrated human blood.

DNA analyses

Restriction endonuclease digestions and other common DNA manipulations were performed by standard procedures as recommended by the manufacturer. Enzymes and DNA ladders were purchased from New England Biolabs. DNA fragments were separated by electrophoresis in horizontal slab gels containing 1% agarose and run in Tris-acetate buffer. DNA was visualized with ethidium bromide.

PCR conditions

Amplification reactions were carried out on total DNA extracted from *H. pylori* in a Perkin Elmer thermal cycler by using a pair of oligonucleotides that match regions near the end of the PPK gene. The predicted product is 1890 base pairs and contains a *Bgl*II site at the midpoint. Primer 1F is a 24mer: CGA AGC CAA AGA TGA GAG CTT GCC. Primer R1W is a 24mer: GCC TTT AGA ATT TAA CTC GTA GCG. Reactions were cycled 25 times; 94°C 1 minute, 52°C 55 seconds, 72°C for 2 minutes plus 7 seconds each cycle.

Insertional Mutagenesis

The PPK PCR product was ligated into pGEM-T-easy, and used to transform *E. coli* DH5-α . Transformants were selected for grown on ampicillin, and disruption of lac-Z, producing white colonies on IPTG and x-gal. Replicate plasmids were harvested (pJWH001, pJWH002, pJWH003, pJWH005,

pJWH006), and cut with *Eco*RI to confirm the insert size. Plasmid pJWH001 was chosen and cut with *Bgl*II in the middle of the PPK insert.
The kanamycin resistance cassette was taken from pILL600 (Labigne, Courcoux, and Tompkins 1992; Labigne-Roussel, Courcoux, and Tompkins 1988)) by cutting with *Bam*HI to release the Kan^r fragment with cohesive ends compatible with the *Bgl*II cut site in PPK. The remaining pBR322 backbone would have been split into two compatible *Bam*HI fragments. Possible ligation products would have included a functional beta-lactamase conferring resistance to ampicillin. Therefore a double digest with *Hinc*II was performed to produce blunt end cuts in both the beta-lactamase and the tetracycline resistance genes in the pBR322 backbone. After complete digestion with both enzymes the only possible ligation product conferring resistance to both ampicillin and kanamycin was the desired insertion of the kanamycin resistance cassette into the PPK gene.
The *Bam*HI and *Bgl*II ends were ligated and the resulting plasmid was used to transform *E. coli* DH5-α and selected on ampicillin and kanamycin. Four colonies were picked and replicate plasmids were harvested (pJWH007, pJWH008, pJWH009, pJWH010). The plasmid pJWH008 was used to transform *H. pylori.*

Transformation of H. pylori

Three plates of *H. pylori* type strain (ATCC 43504) were resuspended and washed in ice cold 15% glycerol 7% sucrose wash buffer, in 1 mL, 1mL, 0.5 mL, and resuspended in 0.08 mL and split into 2 prechilled electroporation cuvettes. 40 mL of cells and 2.5 mL plasmid DNA were incubated for 10 minutes on ice, then electroporated at 2.5 kV. Time constants were 5.6 for the first cuvette and 1.9 for the second cuvette. The cells were incubated a further 10 minutes on ice, then plated and grown for two days on TSA plus 5% defibronated sheep blood. All outgrowth was resuspended in 100 mL SOC and a loopfull was streaked in triplicate onto plates containing kanamycin. Two days later, several pinpoint colonies from the kanamycin plates were streaked onto fresh kanamycin plates. Five days later two colonies were chosen (JWH001, JWH002), grown without kanamycin and frozen at -80C in brucella broth with 20% glycerol. The mutant construction was verified with PCR (Figure 1.) using the same primers and conditions used for cloning. Biochemical characterization was performed with the assistance of the Arthur Kornberg lab, Stanford University School of Medicine, as detailed in appendix 1.

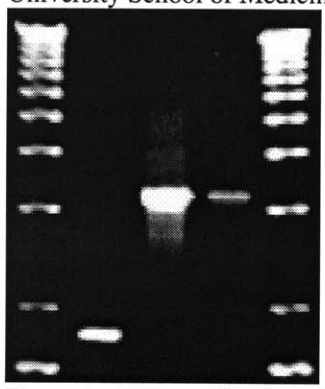

Figure 1. PCR verification of isogenic PPK mutant construction. PCR was performed on genomic DNA. Lanes 1 and 5: 1 kb DNA ladder. Lane 2: type strain. Lane 3: JWH001. Lane 4: JWH002.

Storage and CFU Survival, experiment 1

The *H. pylori* type strain and the JWH002 derivative were grown overnight in 10 mL OB stirred in a 25 mL flask. The cultures were diluted by mixing 0.4 mL culture and 3.6 mL distilled water into a 5 mL snap cap tube and placed at 4°C. At 0, 5, 9, 19, 24, and 30 days, serial dilutions in phosphate buffered saline (PBS) were plated by spreading 0.1 mL samples onto TSA + 5% defibronated sheep blood. Plates were placed inside anaerobic jars. The jars were twice evacuated to 80 kPA and filled with 10% H_2, 10% CO_2, and 80% N_2. The jars were placed inside a 37°C incubator. Colonies were counted after 3 to 5 days growth.

Storage and CFU Survival, experiment 2

The *H. pylori* N6 strain, and its derivative ureB mutant N6::ureB^Kan (courtesy of Jay Solnick; Ferrero et all, 1992), constructed with the same kanamycin resistance cassette from pILL600 were used to control for the survival effects of the kanamycin resistance gene. The *H. pylori* type strain, its JWH001 derivative, the *H. pylori* N6 strain, and its N6::ureB^Kan derivative were grown overnight unstirred in 10 mL OB in a culture tube. The upper 8.5 mL of culture was harvested and the cells were pelleted. The supernatant was carefully removed and 1 mL was mixed with 9 mL distilled water. 3 mL of diluted supernatant was used to resuspend the cell pellets. 1 mL aliquots were placed in 1.5 mL conical snap top centrifuge tubes, briefly centrifuged and placed at 4°C. At 0, 2, and 5 days, samples were withdrawn, vortexed for 10s, and serial dilutions in PBS were plated by spreading 0.1 mL samples onto TSA + 5% defibronated sheep blood. Plates were placed in a 5% $C0_2$ incubator at 37°C. Colonies were counted after 4 or 5 days. Colonies were counted after 4 or 5 days.

Storage and CFU Survival, experiment 3

The *H. pylori* G27 strain, and its derivative PPK mutant G27:PPK^Cam (courtesy of Timothy McDaniels) were harvested from one day old plates with a plastic scraper and placed in 50 mL Tryptic Soy Broth (TSB) supplemented with 5% heat inactivated calf serum in a 250 mL culture flask with a magnetic stir bar. The flasks were placed inside anaerobic jars. The jars were twice evacuated to 80 kPA and filled with 10% H_2, 10% CO_2, and 80% N_2. The assemblies were placed on a multi-channel stir plate inside a 37°C incubator. After 5 days of growth 10 mL of culture was removed and added to fresh media and the new flasks were assembled gassed and incubated as before. After 3 days of secondary broth growth, 1 mL aliquots were placed in 1.5 mL conical snap top centrifuge tubes, briefly centrifuged and placed at 4°C . At 0, 3, 5, 6, 11, 12, and 18 days, samples were withdrawn, vortexed for 10s, and serial dilutions in PBS were plated by drip spreading 0.1 mL samples onto TSA + 5% defibronated sheep blood. Plates were set in a 5% $C0_2$ incubator at 37°C. Colonies were counted after 3 to 5 days growth.

Results

PP and PPK Biochemical assays results

Compared to wild type JWH001 showed a less than 20% reduction in accumulated PP levels. PPK activity was uniformly very low, less than 250 picomol/mg/minute. Background radiation was 204 cpm, while the highest sample was only 549 cpm. Therefore, the signal was barely above twice the background noise and the assay should be repeated with a higher specific radioactivity.

The activity results agree generally with those of the Kornberg lab on other *Helicobacter* strains. They also found very low specific activities despite large accumulations of PP. The mutant strain used in experiment 3 when assayed by the Kornberg lab showed a ~99% reduction in accumulated PP (Cresson Fraley, personal communication).

Storage and CFU Survival, experiment 1 results

Experiment 1 measured the long term CFU survival of the type strain of *H. pylori* and my isogenic PPK mutant constructed with a kanamycin resistance marker. The mutant did not survive as well as the wild type. Starting with concentrations of a little more than 10^8 CFU/mL, both strains survived for at least 24 days. The death curve was nearly exponential. Between 9 and 24 days the PPK mutant lost culturability more quickly. At 24 days the overall fraction surviving was about 60 times higher in the wild type than the mutant, suggesting that PPK was involved in long term survival.

However, the effect measured in experiment 1 could also have been caused by the kanamycin resistance marker. The resistance is achieved with a membrane spanning pump which removes kanamycin molecules from the cell. This may disrupt the normal membrane enough to cause the cells to die more quickly in death phase. Because this experiment lacked any control for the kanamycin marker, the results were not conclusive.

Storage and CFU Survival, experiment 2 results

Experiment 2 measured the long term CFU survival of 4 strains of *H. pylori* : 1) the type strain, 2) its isogenic PPK^aphA-3 mutant, 3) the N6 strain, and 4) its isogenic ureB^aphA-3 mutant. The strains were chosen based on the assumption that under the tested conditions urease activity has no effect on long term survival. Any difference between the two N6 strains should be ascribed to the kanamycin resistance marker. A comparison of the differences within each pair of strains would reveal the extent to which the difference in survival in the PPK pair was due to the disruption of the PPK gene and the extent to which it was due to the kanamycin resistance marker.

The starting concentration in experiment 2 was around 10^6 CFU/mL. All the cells died rapidly, with no CFUs surviving beyond 5 days. In this brief time, the PPK pair lost culturability at about the same rate. However within the ureB pair the mutant died more quickly than the wild type. Experiment 2 did not achieve a high enough cell density nor did it prolong CFU survival long enough to be compared with the other experiments. Furthermore, the small number of time points sampled makes this experiment unhelpful.

Storage and CFU Survival, experiment 3 results

Experiment 3 measured the long term CFU survival of the G27 strain of *H. pylori* and an isogenic PPK mutant constructed with a chloramphenicol resistance marker. The mutant survived as well as the wild type. Starting with concentrations of a little more than 10^8 and 10^9 CFU/mL, both strains survived for 5 days with almost no deaths. The death curve was not exponential. Between 5 and 11 days both strains lost culturability quickly. The overall shape of the death curves was the same, suggesting that PPK was not involved in long term survival.

Experiment 3 used a chloramphenicol marker and showed no difference in the rate of loss of culturability between wild type and a strain which makes less than 1% (Cresson Fraley, personal communication) of the wild type level of PP. However, the starting CFU concentration was 10 fold different. I conclude therefore that I have not demonstrated a role for polyphosphate in the long term survival of *Helicobacter pylori.*

Discussion

The results of my experiments were equivocal. In order to conclusively determine that PPK does not play a role in long term survival of *H. pylori*, a different set of experiments must be performed. If an unmarked deletion of the PPK gene could be constructed, there would be no need for an extra strain to control for the effect of the antibiotic resistance gene. Otherwise, a set of isogenic mutants must be created from a single parent *H. pylori* strain, in which the antibiotic resistance marker has been inserted into the PPK gene in one mutant and in other mutants into a few other places where it either doesn't interrupt a gene or interrupts a gene known to have no role in long term survival.

Furthermore, the experiments should be repeated several times under the same conditions, with more replicate samples plated at each time point. If these conditions can be met, a role for PPK in the long term survival of *H. pylori* can be definitively disproved or proven.

Result data in table and graph form

Day	Exp 1 Type	Exp 1 Type: PPK/Kan	Exp 2 Type	Exp 2 Type: PPK/Kan	Exp 2 N6	Exp 2 N6: ureB/Kan	Exp 3 G27	Exp 3 G27: PPK/Cam
0	**3.1 E 8**	**2.0 E 8**	**7.0 E 5**	**1.1 E 5**	**2.7 E 5**	**1.9 E 6**	**1.1 E 9**	**2.0 E 8**
2			**1.0 E 3**	**3.0 E 1**	**9.1 E 3**	**8.0 E 1**		
3							**8.0 E 8**	**1.5 E 8**
5	**1.3 E 7**	**5.0 E 6**			**6.0 E 1**		**3.2 E 8**	**2.7 E 7**
6							**1.6 E 8**	**2.4 E 6**
9	**5.0 E 6**	**1.4 E 6**						
11							**1.0 E 3**	**9.0 E 0**
12							**3.3 E 2**	
18							**9.0 E 0**	
19	**1.4 E 5**	**1.7 E 3**						
24	**2.5 E 4**	**2.5 E 2**						
30	**4.4 E 3**							

Table 1. Colony forming units per mL

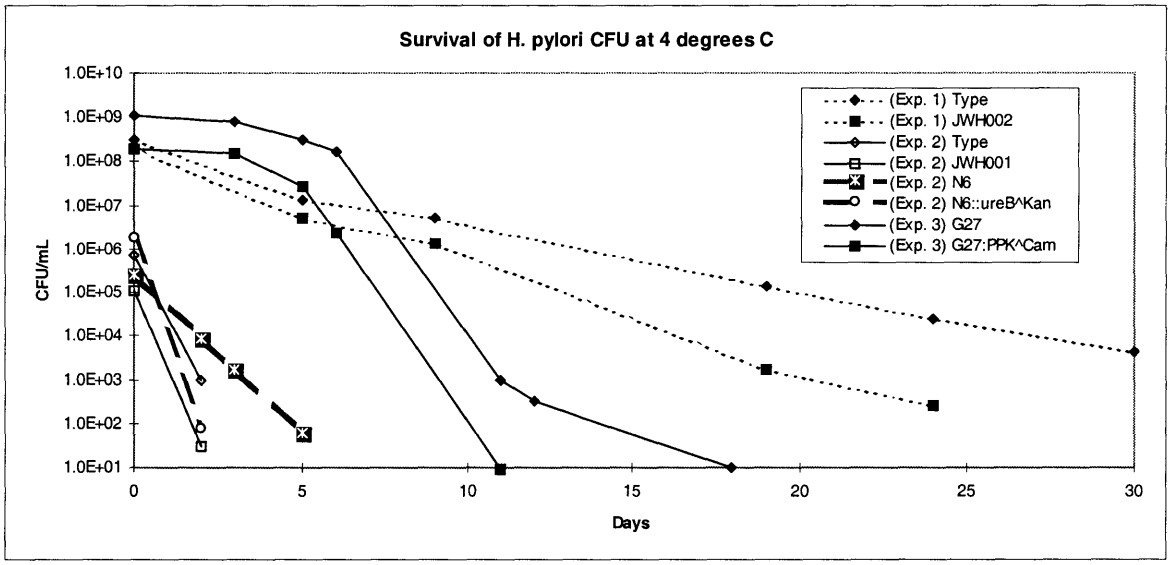

Figure 2. The decline in culturability is shown as colony forming units per mL at each sampling time. Data points in experiment 1 represents a single sampling. Data points in experiments 2 and 3 represent the mean of three independent samplings. The final points on experiment 3 were below the limit of detection (10 CFU/mL).

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Appendix 1

Polyphosphate biochemical assays

These assays were developed in the Arthur Kornberg lab, Department of Biochemistry, Stanford University School of Medicine, Stanford California. These assays were performed in their lab with their gracious help. The assays were performed on plate grown bacteria, not broth grown cultures. This is known to be problematic because the cells are in different growth phases.

Glass Milk Polyphosphate Extraction from Cells

1st cell lysis: Cell pellet + 300 microliters 4 M GITC, 50 mM Tris. Resuspend. Incubate 3 minutes at 95°C. Vortex for 5 seconds at 1 minute and at the end.

Protein denaturation: Add 30 microliters 10% SDS. Incubate 2 minutes at 95°C. Should be clear.

PolyP binding: Add 5 microliters glass-milk, 300 microliters 50% ethanol. Vortex 5 seconds. Incubate 30 seconds at 95°C .

Debris discard: Centrifuge 1 minute 1,200 RPM. May have jelly on top. Discard supernatant.

1st wash: Add 200 microliters ice cold wash buffer (50 mM NaCl, 5 mM Tris, 5 mM EDTA and 50% ethanol). Vortex 5 seconds, it may be hard to suspend. Centrifuge 30 seconds 1,200 RPM. Discard supernatant.

N. A. digestion: Add 100 microliters digestion mixture (5ug/mL DNAse/ RNAse, 50 mM Tris-HCl pH 7.0 and 5 mM Mg^{2+}. Pipette up and down. Incubate 30 minutes 37°C .

2nd wash: Add 200 microliters ice cold wash buffer (50 mM NaCl, 5 mM Tris, 5 mM EDTA and 50% ethanol). Vortex 5 seconds, it should be easy to suspend. Centrifuge 30 seconds 1,200 RPM. Discard supernatant.

3rd wash: Add 200 microliters ice cold wash buffer (50 mM NaCl, 5 mM Tris, 5 mM EDTA and 50% ethanol). Vortex 5 seconds, it should be easy to suspend. Centrifuge 30 seconds 1,200 RPM. Discard supernatant.

1st polyP extraction: Add 100 microliters dH_2O. Vortex briefly. Incubate 30 seconds 95°C . Vortex briefly. Centrifuge 1 minute 1,200 RPM. Save supernatant.

2nd polyP extraction: Add 100 microliters dH_2O. Vortex briefly. Incubate 30 seconds 95°C . Vortex briefly. Centrifuge 1 minute 1,200 RPM. Save supernatant, pool with 1st extraction.

PolyP storage: at -20°C .

Polyphosphate Detection

ATP production: Mix a reaction tube with 10 microliters of polyP sample and final concentrations: 50 mM HEPES-KOH (pH 7.2), 40 mM ammonium sulfate, 4 mM $MgCl_2$, 0.1 mM ADP, 10-50 ng PPK. Incubate 40 minutes 37°C . Stop the reaction by adding 5 mM EDTA, 50 mM Tris total.

ATP detection: According to the luciferase kit manufacturer's directions.

Polyphosphate Kinase Activity Sample Preparation

A cell pellet equivalent to 1 mL culture is suspended in 1 mL Tris-HCl buffer (50 mM pH7.5) containing 10% sucrose. Freeze in liquid nitrogen and store at -80°C if not used immediately.

To cell suspension add 2mM fresh dithiothreitol and 250 microgram/mL lysozyme. Incubate at 0°C for 30 minutes. Lyse cells for 4 minutes in a 37°C water bath, followed by immediate chilling in an ice bath. Sonicate in an ice bath for 30 seconds. Centrifuge 5 minutes at 5000 RPM at 4°C in a microfuge. Discard the pellet, which contains unbroken cells and cell wall fragments. Centrifuge the low speed

supernatant in a table-top Beckman ultra centrifuge 100.2 rotor for 10 minutes at 95,000 RPM (or 30 minutes at 50,000 RPM) at 4°C . Save both the pellet and the supernatant.

To the pellet add 100 microliters tris-HCl buffer (pH7.5; 50 mM containing 10% sucrose; 5 mM $MgCl_2$, and 10 microgram/mL each of DNAse I and RNAse A) and sonicate in an ice bath 3x30 seconds, carefully mixing without vortexing. This is the membrane preparation. Both the membrane preparation and the high speed supernatant can be tested for polyphosphate kinase and exopolyphosphatase I activity.

Polyphosphate Kinase Activity Assay

The assay measures the formation of the acid-insoluble [^{32}P] polyphosphate by the polymerization of the terminal phosphate [gamma-^{32}P] of ATP.

Reaction Mixture: hepes-KOH buffer pH 7.2 50 mM, ammonium sulfate 40 mM, $MgCl_2$ 4mM, [gamma-^{32}P] ATP (2000 cpm/nmol) 1mM, creatine phosphate 2mM, creatine kinase 20 micrograms/mL, in a total volume with the samle of 250 microliters.

PPK is added after the reaction mix is equilibrated to 37°C . Control tubes are set up with heat inactivated enzyme and/or with enzyme buffer.

After incubation, the reaction is stopped by the addition of 250 microliters of 7% $HClO_4$ and 50 microliters of 2 mg/mL bovine serum albumin. Place the tubes on ice until the contents are filtered. Filter the mixture on presoaked (in washing solution) Whatman GF/C glass fiber filters using vacuum. Wash 3 times with 10 mL (each time) washing solution (0.1 M sodium pyrophosphate made in 1 M HCl) followed by washing 3 times with 10 mL of ehanol (100%), each time. Dry the filters and the [^{32}P]polyP collected on the filters is measured in a liquid scintillation counter. Use 4 mL of the Ready safe scintillation cocktail per filter. Reaction mix can be made at 5X concentration. When a much higher specific activity of ATP (e.g. 10^4 cpm/pmol) is required, excess unlabeled ATP is added after acidification, and the product is washed using a large volume of washing solution to reduce the background (ca. 300 cmp). An ATP regenerating system is used in the assay because of the inhibitory effect of ADP.

Effect of enzyme (protein) concentration: Include various amounts of enzyme based on protein concentration (2-50 micrograms per tube in crude extracts) and by dilution of the enzyme preparation into the reaction mix and incubate for a specific period of time (e.g. 15 minutes) and determine the enzyme activity. Plot activity -vs- protein concentration.

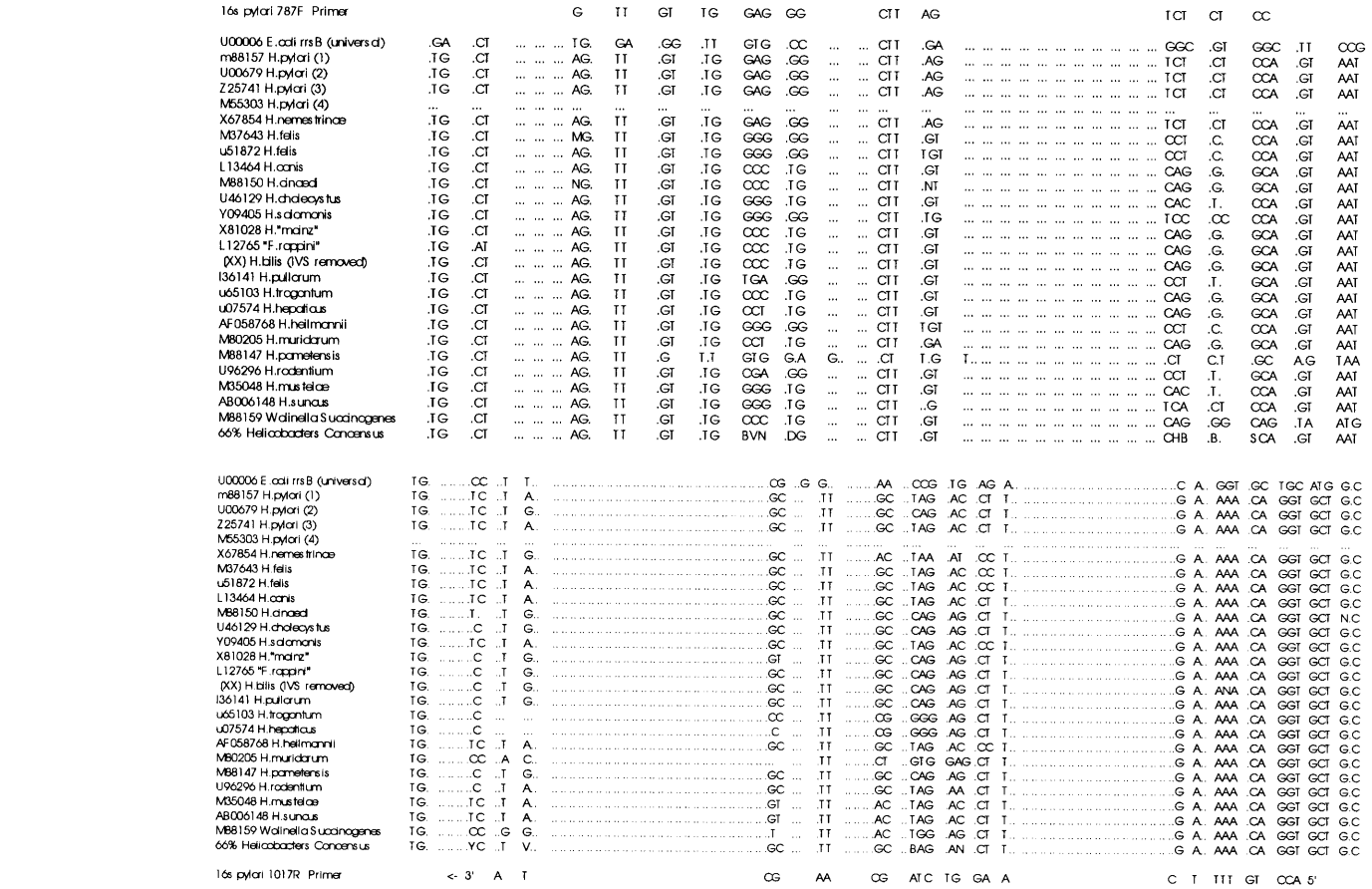
Time course of enzyme reaction: Incubate the reaction mix with a known amount of enzyme, remove aliquots at 0, 5, 10, 15, 20, 30, and 60 minute time intervals and stop reaction by pipetting into 250 microliters of $HClO_4$ (7%). Add 50 microliters of 2 mg/mL BSA, filter and count. Plot activity -vs- time.

Specific activity: One unit of enzyme is defined as the amount incorporating 1 pmol of phosphate into acid-insoluble polyP per minute.

Appendix 2

16s rRNA Primers for Helicobacter detection, differentiation, and quantitation.

The published 16s rRNA sequences for several *Helicobacter* species and the *E. coli* universal sequence were aligned. The 66% consensus sequence was constructed. Primer sites suitable for *H. pylori* specific, and *Helicobacter* genus specific amplification were identified. The primers were extended on the 5' end so as to bring all the predicted melting temperatures close to 60°C



16s pylori 976F Primer		G	AAA	T	AG	TG	GAG	TG	TC	T	A	GC	TT	GC	TAG	AC
U00006 E.coli rrsB (universal)	TCA	..G	AGA	..T	GA.	G.A	ATG	...TG.	CC	..T	T..	.....CG	..G G..	.....AA	... CCG	.TG .AG A..
m88157 H.pylori (1)	CTA	..G	AAA	..T	AG.	T.G	GAG	...TG.	.....TC	..T	A.	.....GC	... .TT	.....GC	... TAG	.AC .CT T..
U00679 H.pylori (2)	CTA	..G	AAA	..T	AG.	T.G	GAG	...TG.	.....TC	..T	G.	.....GC	... .TT	.....GC	... CAG	.AC .CT T..
Z25741 H.pylori (3)	CTA	..G	AAA	..T	AG.	T.G	GAG	...TG.	.....TC	..T	A.	.....GC	... .TT	.....GC	... TAG	.AC .CT T..
M55303 H.pylori (4)	...	...	...	..	...	...	...	...	.....	...	...	...	... ...	.....	... ...	... ...
X67854 H.nemestrince	CTA	..G	AAA	..T	AG.	T.G	GAG	...TG.	.....TC	..T	G.	.....GC	... .TT	.....AC	... TAA	.AT .CC T..
M37643 H.felis	CTA	..G	AAA	..T	AG.	G.G	GAG	...TG.	.....TC	..T	A.	.....GC	... .TT	.....GC	... TAG	.AC .CC T..
u51872 H.felis	CTA	..G	AAA	..T	AG.	G.G	GAG	...TG.	.....TC	..T	A.	.....GC	... .TT	.....GC	... TAG	.AC .CC T..
L13464 H.canis	CTA	..G	AGA	..T	AG.	C.G	GAG	...TG.	.....TC	..T	A.	.....GC	... .TT	.....GC	... TAG	.AC .CT T..
M88150 H.cinaed	CTA	..G	AGA	..T	AG.	T.G	GAG	...TG.	.....T.	..T	G.	.....GC	... .TT	.....GC	... CAG	.AG .CT T..
U46129 H.cholecystus	CTA	..G	AGA	..T	AG.	C.G	GAG	...TG.	C	..T	G.	.....GC	... .TT	.....GC	... CAG	.AG .CT T..
Y09405 H.salomonis	CTA	..G	AAA	..T	AG.	C.G	GAG	...TG.	.....TC	..T	A.	.....GC	... .TT	.....GC	... TAG	.AC .CC T..
X81028 H."mainz"	CTA	..G	AGA	..T	AG.	C.G	GAG	...TG.	C	..T	G.	.....GT	... .TT	.....GC	... CAG	.AG .CT T..
L12765 "F.rappini"	CTA	..G	AGA	..T	AG.	T.G	GAG	...TG.	C	..T	G.	.....GC	... .TT	.....GC	... CAG	.AG .CT T..
(XX) H.bilis (IVS removed)	CTA	..G	AGA	..T	AG.	T.G	GAG	...TG.	C	..T	G.	.....GC	... .TT	.....GC	... CAG	.AG .CT T..
I36141 H.pullorum	CTA	..G	AGA	..T	AG.	C.G	GAG	...TG.	C	..T	G.	.....GC	... .TT	.....GC	... CAG	.AG .CT T..
u65103 H.trogontum	CTA	..G	AGA	..T	AG.	C.G	GAG	...TG.	C	...	...	CC	... .TT	.....CG	... GGG	.AG .CT T..
u07574 H.hepaticus	CTA	..G	AGA	..T	AG.	T.G	GAG	...TG.	C	...	...	C	... .TT	.....CG	... GGG	.AG .CT T..
AF058768 H.heilmannii	CTA	..G	AAA	..T	AG.	C.G	GAG	...TG.	.....TC	..T	A.	.....GC	... .TT	.....GC	... TAG	.AC .CC T..
M80205 H.muridarum	ATA	..G	AGA	..T	AT.	G.G	GAG	...TG.	CC	..A	C.	.....	... .TT	.....CT	... GTG	GAG .CT T..
M88147 H.pametensis	CTA	..G	AGA	..T	AG.	C.G	GAG	...TG.	C	..T	G.	.....GC	... .TT	.....GC	... CAG	.AG .CT T..
U96296 H.rodentium	CTA	..G	AGA	..T	AG.	T.G	GAG	...TG.	C	..T	A.	.....GC	... .TT	.....GC	... TAG	.AA .CT T..
M35048 H.mustelae	CTA	..G	AAA	..T	AG.	C.G	GAG	...TG.	.....TC	..T	A.	.....GT	... .TT	.....AC	... TAG	.AC .CT T..
AB006148 H.suncus	CTA	..G	AGA	..T	AG.	T.G	GAG	...TG.	.....TC	..T	A.	.....GT	... .TT	.....AC	... TAG	.AC .CT T..
M88159 Wolinella Succinogenes	ATA	..G	AGA	..T	AT.	G.G	GAG	...TG.	CC	..G	G.	.....T	... .TT	.....AC	... TGG	.AG .CT T..
66% Helicobacters Concensus	CTA	..G	ARA	..T	AG.	B.G	GAG	...TG.	.....YC	..T	V.	.....GC	... .TT	.....GC	... BAG	.AN .CT T..

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U00006 E.coli rrsB (universal)	G.C	GG.	.....	...	TCC	GG.	...	...	...	.CC	GGG	.AA	.CT	.C.	A.A	AG.	GA.	GA.	CT.	G.C
m88157 H.pylori (1)	T.G	CT.	.....	..A	ACA	GG.	...	...	TTA	TG.	CTG	AG.	AA.	CT.	C.T	.AA	G.G	A.T	A.C	T.G	.CC
U00679 H.pylori (2)	T.G	CT.	.....	..A	ACA	GG.	...	...	TCA	TG.	CTG	AG.	AA.	CT.	C.T	.AA	G.G	A.T	A.C	T.G	.CC
Z25741 H.pylori (3)	T.G	CT.	.....	..A	ACA	GG.	...	...	TTA	TG.	CTG	AG.	AA.	CT.	C.T	.AA	G.G	A.T	A.C	T.G	.CC
M55303 H.pylori (4)	...	...	.....	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
X67854 H.nemestrince	T.G	CT.	.....	..A	ACA	GG.	...	...	.CA	TG.	CTG	AG.	AA.	CT.	C.T	.AA	G.G	A.T	A.C	T.G	.CC
M37643 H.felis	T.G	CT.	.....	..A	ACA	GG.	...	...	TTN	TG.	CTG	AG.	CT.	CT.	C.T	.AA	G.A	A.T	A.C	T.G	.CC
u51872 H.felis	T.G	CT.	.....	..A	ACA	GG.	...	...	TNN	TG.	CTG	AG.	CT.	CT.	C.T	.AA	G.A	A.T	A.C	T.G	.CC
L13464 H.canis	T.G	CT.	.....	..A	GCA	G..	...	...	TTC	GG.	CTG	AG.	CA.	CT.	C.T	.AA	G.G	A.G	A.C	T.G	.CC
M88150 H.cinaed	T.G	CT.	.....	..A	GCA	G..	...	...	CTC	GG.	CTG	AG.	CA.	CT.	C.T	.AA	G.G	A.G	A.C	T.G	.CC
U46129 H.cholecystus	T.G	CT.	.....	..A	GCA	G..	...	...	TTC	GG.	CTG	AG.	CA.	CT.	C.T	.AG	G.G	A.G	A.C	T.G	.CC
Y09405 H.salomonis	T.G	CT.	.....	..A	ACA	GG.	...	...	TAG	TG.	CTG	AG.	CT.	CT.	C.T	.AA	G.A	A.T	A.C	T.G	.CC
X81028 H."mainz"	T.G	CT.	.....	..A	GCA	G..	...	...	TTC	GG.	CTG	AG.	CA.	CT.	C.T	.AA	G.G	A.G	A.C	T.G	.CC
L12765 "F.rappini"	T.G	CT.	.....	..A	GCA	G..	...	...	TTC	GG.	CTG	AG.	CA.	CT.	C.T	.AA	G.G	A.G	A.C	T.G	.CC
(XX) H.bilis (IVS removed)	T.G	CT.	.....	..A	GCA	G..	...	...	TTC	GG.	CTG	AG.	CA.	CT.	C.T	.AA	G.G	A.G	A.C	T.G	.CC
I36141 H.pullorum	T.G	CT.	.....	..A	GCA	G..	...	...	TTC	GG.	CTG	AG.	CA.	CT.	C.T	.AA	G.G	A.G	A.C	T.G	.CC
u65103 H.trogontum	T.G	CT.	.....	..A	GCA	G..	...	...	TTC	GG.	CTG	AG.	CA.	CT.	C.T	.AA	G.G	A.G	A.C	T.G	.CC
u07574 H.hepaticus	N.N	NN.	.....	..N	NNN	NN.	...	...	NNN	NN.	NNN	NN.	NN.	NN.	N.N	.NN	N.N	N.N	N.N	N.N	.NN
AF058768 H.heilmannii	T.G	CT.	.....	..A	ACA	GG.	...	...	TAR	TG.	CTG	AG.	CT.	CT.	C.T	.AA	G.A	A.G	A.C	T.G	.CC
M80205 H.muridarum	T.G	CT.	.....	..A	GCA	GT.	...	...	TTN	AG.	CTG	AG.	CA.	CT.	C.T	.AA	G.G	A.G	A.C	T.G	.CC
M88147 H.pametensis	T.G	CT.	.....	..A	ACA	G..	...	...	TTC	GG.	CTG	AG.	CA.	CT.	C.T	.AG	G.G	A.G	A.C	T.G	.CC
U96296 H.rodentium	T.G	CT.	.....	..A	ACT	A..	...	...	TTC	GG.	TAG	AG.	CA.	CT.	C.T	.AA	G.G	A.G	A.C	T.G	.CC
M35048 H.mustelae	T.G	CT.	.....	..A	GCA	G..	...	...	TTC	GG.	CTG	AG.	CA.	CT.	C.T	.AA	G.A	A.G	A.C	T.G	.CC
AB006148 H.suncus	T.G	CT.	.....	..A	GCA	GT.	...	...	.TC	GG.	CTG	AG.	CA.	CT.	C.T	.AA	G.A	A.G	A.C	T.G	.CC
M88159 Wolinella Succinogenes	T.G	CT.	.....	..A	ACG	G..	...	...	TTC	GG.	CCG	AG.	CA.	CT.	C.T	.AG	T.G	A.G	A.C	T.G	.CC
66% Helicobacters Concensus	T.G	CT.	.....	..A	NCA	GN.	...	...	TTN	NG.	CTG	AG.	CA.	CT.	C.T	.AA	G.G	A.N	A.C	T.G	.CC

16s pylori 1017R Primer		<-	3'	T	TGT	CC	AAT	AC	GAC	TC	TT	GA	GA	TT	CC	TA	TG	5'

16s pylori 212F Primer (general)		G	ATC		AG	CCT	AT	G	T	C	CT	AT	CA	GC	TT	G	TT		
U00006 E.coli rrsB (universal)	TCG	G..	ATG	...	T.G	CCC	AG.	...A	T..	..G	GG.	.AT	TA.	G.C	TA.	G..	.TA	GG.	
m88157 H.pylori (1)	AGA	G..	ATC	...	A.G	CCT	AT.	...G	T..	..C	CT.	.AT	CA.	G.C	TT.	G..	.TT	GG.	
U00679 H.pylori (2)	AGA	G..	ATC	...	A.G	CCT	AT.	...G	T..	..C	CT.	.AT	CA.	G.C	TT.	G..	.TT	GG.	
Z25741 H.pylori (3)	AGA	G..	ATC	...	A.G	CCT	AT.	...G	T..	..C	CT.	.AT	CA.	G.C	TT.	G..	.TT	GG.	
M55303 H.pylori (4)	AGA	G..	ATC	...	A.G	CCT	AT.	...G	T..	..C	CT.	.AT	CA.	G.C	TT.	G..	.TT	GG.	
X67854 H.nemestrince	GAG	G..	ATC	...	A.G	CCT	AT.	...G	T..	..C	CT.	.AT	CA.	G.C	TT.	G..	.TT	GG.	
M37643 H.felis	AAG	G..	ATT	...	G.G	TCT	AT.	...G	T..	..C	CT.	.AT	CA.	G.C	TT.	G..	.TT	GG.	
u51872 H.felis	AAG	G..	ATT	...	G.G	TCT	AT.	...G	T..	..C	CT.	.AT	CA.	G.C	TT.	G..	.TT	GG.	
L13464 H.canis	AAG	G..	ATC	...	A.G	CCT	AT.	...G	T..	..C	CT.	.AT	CA.	G.C	TT.	G..	.TT	GG.	
M88150 H.cinaed	.AA	GG.	.AT	C..	.A.	GCC	TAT	... ...	GT.	...	CCT	..A	TCA	.G.	CTT	.G.	.T	TGG	
U46129 H.cholecystus	AAG	G..	ATC	...	A.G	CCT	AT.	...G	T..	..C	CT.	.AT	CA.	G.C	TT.	G..	.TT	GG.	
Y09405 H.salomonis	AAG	G..	ATT	...	G.G	TCT	AT.	...G	T..	..C	CT.	.AT	CA.	G.C	TT.	G..	.TT	GG.	
X81028 H."mainz"	TGG	G..	ATT	...	A.G	CCT	AT.	...G	T..	..C	CT.	.AT	CA.	G.C	TT.	G..	.TT	GG.	
L12765 "F.rappini"	...	...	...	...	...	...	...	... ...	...	...	...	...	...	...	...	...	...	...	
(XX) H.bilis (IVS removed)	AAG	G..	ATC	...	A.G	CCT	AT.	...G	T..	..C	CT.	.AT	CA.	G.C	TT.	G..	.TT	GG.	
I36141 H.pullorum	AGG	G..	ATT	...	G.G	CCT	AT.	...G	T..	..C	CT.	.AT	CA.	G.C	TT.	G..	.TT	GG.	
u65103 H.trogontum	TGG	G..	ATT	...	G.G	CCT	AT.	...G	T..	..C	CT.	.AT	CA.	G.C	TT.	G..	.TT	GG.	
u07574 H.hepaticus	AAG	G..	ATC	...	A.G	CCT	AT.	...G	T..	..C	CT.	.AT	CA.	G.C	TT.	G..	.TT	GG.	
AF058768 H.heilmannii	AAG	G..	ATT	...	G.G	TCT	AT.	...G	T..	..C	CT.	.AT	CA.	G.C	TT.	G..	.TT	GG.	
M80205 H.muridarum	AAG	G..	ATC	...	A.G	CCT	AT.	...G	T..	..C	CT.	.AT	CA.	G.C	TT.	G..	.TT	GG.	
M88147 H.pametensis	TGG	G..	ATC	...	A.G	CCT	AT.	...G	T..	..C	CT.	.AT	CA.	G.C	TT.	G..	.TT	GG.	
U96296 H.rodentium	AAG	G..	ATC	...	A.G	CCT	AT.	...G	T..	..C	CT.	.AT	CA.	G.C	TT.	G..	.TT	GG.	
M35048 H.mustelae	TGG	G..	ATC	...	A.G	CCT	AT.	...G	T..	..C	CT.	.AT	CA.	G.C	TT.	G..	.TT	GG.	
AB006148 H.suncus	TGG	G..	ATC	...	A.G	CCT	AT.	...G	T..	..C	CT.	.AT	CA.	G.T	TT.	G..	.TT	GG.	
M88159 Wolinella Succinogenes	TGG	G..	ATC	...	A.G	CCT	AT.	...G	T..	..C	CT.	.AT	CA.	G.C	TT.	G..	.TT	GG.	
66% Helicobacters Concensus	ARG	G..	ATY	...	A.G	CCT	AT.	...G	T..	..C	CT.	.AT	CA.	G.C	TT.	G..	.TT	GG.	

U00006 E.coli rrsB (universal)	.T	C.C	CT.	AG.	C.T	G.G	.T.C	T..	G.A	GA.	GGA	T..	
m88157 H.pylori (1)	.C	G.G	GT.	AT.	C.C	G.G	.CC	T..	G.A	GA.	GGG	T..	
U00679 H.pylori (2)	.C	G.G	GT.	AT.	C.C	G.G	.CC	T..	G.A	GA.	GGG	T..	
Z25741 H.pylori (3)	.C	G.G	GT.	AT.	C.C	G.G	.CC	T..	G.A	GA.	GGG	T..	
M55303 H.pylori (4)	.C	G.G	GT.	AT.	C.C	G.G	.CC	T..	G.A	GA.	GGG	T..	
X67854 H.nemestrinae	.C	G.G	GT.	AT.	C.C	G.G	.CC	T..	G.A	GA.	GGG	T..	
M37643 H.felis	.C	G.G	GT.	AT.	C.C	G.G	.CC	T..	G.A	GA.	GGG	T..	
u51872 H.felis	.C	G.G	GT.	AT.	C.C	G.G	.CC	T..	G.A	GA.	GGG	T..	
L13464 H.canis	.C	G.G	GT.	AT.	C.C	G.G	.CC	T..	G.A	GA.	GGG	T..	
M88150 H.cinaed	..C	G.G	GT.	AT.	C.C	G.G	.CC	T..	G.A	GA.	GGG	T..	
U46129 H.cholecystus	.C	G.G	GT.	AT.	C.C	G.G	.CC	T..	G.A	GA.	GGG	T..	
Y09405 H.salomonis	.C	G.G	GT.	AT.	C.C	G.G	.CC	T..	G.A	GA.	GGG	T..	
X81028 H."mainz"	.C	G.G	GT.	AT.	C.C	G.G	.CC	T..	G.A	GA.	GGG	T..	
L12765 "F.rappini"	..	...	...	...	...	...	...	...	...	...	...	...	
(XX) H.bilis (IVS removed)	.C	G.G	GT.	AT.	C.C	G.G	.CC	T..	G.A	GA.	GGG	T..	
I36141 H.pullorum	.C	G.G	GT.	AT.	C.C	G.G	.CC	T..	G.A	GA.	GGG	T..	
u65103 H.trogontum	.C	G.G	GT.	AT.	C.C	G.G	.CC	T..	G.A	GA.	GGG	T..	
u07574 H.hepaticus	.C	G.G	GT.	AT.	C.C	G.G	.CC	T..	G.A	GA.	GGG	T..	
AF058768 H.heilmannii	.C	G.G	GT.	AT.	C.C	G.G	.CC	T..	G.A	GA.	GGG	T..	
M80205 H.muridarum	.C	G.G	GT.	AT.	C.C	G.G	.CC	T..	G.A	GA.	GGG	T..	
M88147 H.pametensis	.C	G.G	GT.	AT.	C.C	G.G	.CC	T..	G.A	GA.	GGG	T..	
U96296 H.rodentium	.C	G.G	GT.	AT.	C.C	G.G	.CC	T..	G.A	GA.	GGG	T..	
M35048 H.mustelae	.C	G.G	GT.	AT.	C.C	G.G	.CC	T..	N.A	GA.	GGG	T..	
AB006148 H.suncus	.C	G.G	GT.	AT.	C.C	G.G	.CC	T..	G.A	GA.	GGG	T..	
M88159 Wolinella Succinogenes	.C	G.G	GT.	AT.	C.C	G.G	.CC	T..	G.A	GA.	GGG	T..	
66% Helicobacters Concensus	.C	G.G	GT.	AT.	C.C	G.G	.CC	T..	G.A	GA.	GGG	T..	
16s pylori 289R Primer (general) 3'	G	CC	CA	TA	GG	CC	GG	A	CT	CT	CCC	A	5'

U00006 E.coli rrsB (universal)	..C	A..	GGT	.GC	TGC	ATG	G.C	TG.	TCG
m88157 H.pylori (1)	..G	A..	AAA	.CA	GGT	GCT	G.C	AC.	GGC
U00679 H.pylori (2)	..G	A..	AAA	.CA	GGT	GCT	G.C	AC.	GGC
Z25741 H.pylori (3)	..G	A..	AAA	.CA	GGT	GCT	G.C	AC.	GGC
M55303 H.pylori (4)	~~~	~~~	~~~	~~~	~~~	~~~	~~~	~~~	~~~
X67854 H.nemestrinae	..G	A..	AAA	.CA	GGT	GCT	G.C	AC.	GGC
M37643 H.felis	..G	A..	AAA	.CA	GGT	GCT	G.C	AC.	GGC
u51872 H.felis	..G	A..	AAA	.CA	GGT	GCT	G.C	AC.	GGC
L13464 H.canis	..G	A..	AAA	.CA	GGT	GCT	G.C	AC.	GGC
M88150 H.cinaed	..G	A..	AAA	.CA	GGT	GCT	N.C	AC.	GGC
U46129 H.cholecystus	..G	A..	AAA	.CA	GGT	GCT	G.C	AC.	GGC
Y09405 H.salomonis	..G	A..	AAA	.CA	GGT	GCT	G.C	AC.	GGC
X81028 H."mainz"	..G	A..	AAA	.CA	GGT	GCT	G.C	AC.	GGC
L12765 "F.rappini"	..G	A..	AAA	.CA	GGT	GCT	G.C	AC.	GGC
(XX) H.bilis (IVS removed)	..G	A..	ANA	.CA	GGT	GCT	G.C	AC.	GGC
I36141 H.pullorum	..G	A..	AAA	.CA	GGT	GCT	G.C	AC.	GGC
u65103 H.trogontum	..G	A..	AAA	.CA	GGT	GCT	G.C	AC.	GGC
u07574 H.hepaticus	..G	A..	AAA	.CA	GGT	GCT	G.C	AC.	GGC
AF058768 H.heilmannii	..G	A..	AAA	.CA	GGT	GCT	G.C	AC.	GGC
M80205 H.muridarum	..G	A..	AAA	.CA	GGT	GCT	G.C	AC.	GGC
M88147 H.pametensis	..G	A..	AAA	.CA	GGT	GCT	G.C	AC.	GGC
U96296 H.rodentium	..G	A..	AAA	.CA	GGT	GCT	G.C	AC.	GGC
M35048 H.mustelae	..G	A..	AAA	.CA	GGT	GCT	G.C	AC.	GGC
AB006148 H.suncus	..G	A..	AAA	.CA	GGT	GCT	G.C	AC.	GGC
M88159 Wolinella Succinogenes	..G	A..	AAA	.CA	GGT	GCT	G.C	AC.	GGC
66% Helicobacters Concensus	..G	A..	AAA	.CA	GGT	GCT	G.C	AC.	GGC
16s pylori 1026R Primer (general) 3'	C	T	TTT	GT	CCA	CGA	CG	TG	CCG	5'

The primers are named according to the position of the 5' end of the primer in the *H. pylori* sequence. The sites are predicted to lie on small stem loops on the rRNA. The corresponding sites on the *E. coli* rRNA are as follows:

Helicobacter pylori specific primers:

787F	829->852
1017R	1054->1029
976F	1013->1041
1127R	1158->1135

Helicobacter genus primers:

212F	227->253
289R	304->283
1026R	1064->1045