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Characterization Of Phosphate Uptake System In Helicobacter Pylori

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ABSTRACT

Helicobacter pylori is known to be associated with gastritis, peptic ulcer disease and gastric cancer. Evidence strongly suggests that more than 50% of the population of the world is colonized by *H. pylori* with and without disease symptoms. It is now considered one of the most common human pathogens.

Phosphate is an essential nutrient for all cells and its availability in the environment varies widely. In *H. pylori*, phosphate uptake is dependent upon the pH of medium and the rate of uptake is greatly reduced in acidic environments. HP1491 from ATCC 26695 encodes a putative phosphate permease, which has 56% similarity to phosphate permeases/ion co-transporters from *Saccharomyces cerevisiae*, that also are regulated by phosphate limitation and pH. Mutant strain, HPmt1491, was obtained by insertional inactivation with suicide plasmid pBCα5 that carried PCR product of HP1491 gene.

Comparison of phosphate uptake activity in wild type and mutant strains in neutral and acidic (pH 4.5) media showed that the phosphate permease encoded by HP 1491 is responsible for maintaining the low activity observed during low pH and may have a role in the initial rate of activity and recovery of various rates after the initial burst of activity. Further studies are necessary to completely define the HP1491 gene products role in regulation of phosphate uptake.

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Terminology:

ATCC 26695: wild type *H. pylori* strain

HPmt1491: mutant strain of which HP1491 was disrupted

HP1491: phosphate permease

HP1491 gene: DNA which encodes HP1491

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Chapter One

INTRODUCTION

Helicobacter pylori is a spiral, Gram-negative, microaerophilic bacterium that causes one of the most common infections in humans. *H. pylori* was first identified as a human pathogen by Marshall and Warren in 1982 (Marshall and Warren, 1984).

The epidemiology of *H. pylori*

Epidemiological studies have shown that *H. pylori* infection is ubiquitous, with approximately 50% of the world's population infected with this organism. The prevalence of *H. pylori* infection is similar in males and females and it is believed that once infected, it persists for a lifetime unless antimicrobial therapy is administered (Pounder and Ng 1995). In general, the infection rate in developing countries, like China, Thailand and India, has been shown to be higher (44% -79%) than that in developed countries (19%-57%) such as the United States, the United Kingdom and Australia, (Graham et al. 1991a, b; Mitchell et al. 1992a; Perez et al. 1990; Sitas et al. 1991; Whitaker et al. 1993).

Comparison of the age-stratified prevalence rates from such countries indicated that the difference in prevalence between developed and developing countries relates to the rate of acquisition of *H. pylori* in childhood. For example, approximately 27% of Chinese children fewer than 10 years of age are infected as compared with 4% of Australian children. Beyond this age, the prevalence increases approximately 1% per year

in either country (Mitchell et al. 1992a). Many factors have been reported to influence the level of infection within a population, such as: socioeconomic status, hygiene, sanitation, density of population, educational opportunities, ethnic group, etc.

Transmission of *H. pylori*

Humans appear to be the natural host for *H. pylori* and it has been postulated that *H. pylori* has adapted itself to the ecological niche of the human stomach (Lee and Hazell, 1988). It is probable that ingestion is the most common means of acquiring *H. pylori*. So, based on many groups of research data, person-to-person contact is the most likely mode of transmission. (Berkowicz and Lee 1987, Lamber et al. 1995; Vincent et al. 1994). However, whether *H. pylori* reaches the oral cavity via the fecal-oral or oral-oral route, and whether either of these routes of transmission is important in the spread of *H. pylori*, is still not clear and requires further investigation.

Diseases

It is well established that *H. pylori* plays an important role in chronic gastritis, peptic ulcer disease, gastric cancer and B-cell mucosal-associated lymphoid tissue (MALT) lymphoma (Graham et al. 1992; IARC 1994; Marshall et al. 1985; Parsonnet et al. 1994). Variations in acid output causes colonization of different sites with *H. pylori* (Lee et al. 1995). In patients with a high acid output, the bacteria mainly colonize the antral region, while patients with a normal or low acid output have a widespread gastric inflammation involving both the body of the stomach and the antrum. This results in pangastritis, which often progresses into atrophy and finally intestinal metaplasia

(Marshall et al. 1985). In addition, polymorphisms in interleukin 1 (Bukholm et al. 1997) and blood group types also influence the outcome of infection (Guruge et al. 1998; Heneghan et al. 2000).

Diagnosis of *H. pylori* Infection

Esophagogastro-duodenoscopy with gastric biopsies is the standard method for the diagnosis of active *H. pylori* infection. The characteristic histological appearance of *H. pylori* is the 3.0 x 0.5 μm spiral bacilli located adjacent to the gastric epithelium. Culturing of *H. pylori* from patient specimens can provide diagnostic information about the presence of *H. pylori*, although it is very difficult to isolate *in vitro*. High urease activity is a primary diagnostic characteristic of *H. pylori* colonization. Recently, many reasonably accurate detection assays have become commercially available for clinical use. One of the most promising noninvasive methods is ^{13}C or ^{14}C-labeled urea breathe test. In *H. pylori*-colonized patients, the urea is rapidly metabolized to ammonia and labeled bicarbonate, and the latter is carried to the lungs and excreted in the expired breath as labeled carbon dioxide, which can be quantified. Other noninvasive seroimmunological techniques have been used to determine antibody response to *H. pylori*, such as hemagglutination, bacterial agglutination, complement fixation, immunoblotting, and ELISA. Interestingly, even *H. pylori* has also been detected by PCR from biopsy specimens (Bickley et al. 1993; Kooistra-smid et al. 1993), gastric juice and saliva (Westblom et al. 1993), dental plaque (Bickley et al. 1993; Nguyen et al. 1993), and feces (Mapstone et al. 1993). Due to the relatively high cost and ease of contamination, PCR is not used for routine diagnostic testing.

Virulence factors

Over recent years, a number of putative virulence factors of *H. pylori* have been proposed such as urease, the heat shock protein, vacuolating cytotoxin (*vac*A), the cytotoxin associated gene A (*cag*A) and the cytotoxin associated gene II (*cag* II). Both *in vivo* and *in vitro*, *H. pylori* also produces enzymes such as catalase, oxidase, protease and phospholipase and specific adhesion proteins that enable them to adhere to mucous and epithelial cells.

Colonization of the host

H. pylori produces copious amounts of urease, which has been hypothesized to protect the bacterium by locally neutralizing acid in its microenvironment through hydrolysis of gastric urea to ammonia (Owen et al. 1985). The ammonia provides the bacterium time to safely traverse the mucus layer and colonize the surface of the epithelium.

Motility

H. pylori cells have four or six unipolar flagella (Geis et al. 1989; Suerbaum 1995) that play a role in movement of *H. pylori* from the acidic gastric environment to the more neutral environment of the gastric epithelium. Mutants of *H. pylori* that are nonmotile are unable to colonize and survive in the gnotobiotic piglet (Eaton et al. 1989, 1992, 1996), for this reason flagella have been determined to be a virulence factor for *H. pylori*.

Adhesion molecules

Since the gastric epithelium and mucus are constantly moving, *H. pylori* must have developed mechanisms to keep itself in the gastric mucosal environment. Adhesions from *H. pylori* include: a 29 kDa sialic acid lectin, HpaA, which binds to sialoconjugates (25 kDal) (Evans et al. 1988, 1993) and *H. pylori* outer membrane protein that binds laminin in an α-2,3 sialic acid-dependent manner and requires the presence of *H. pylori* lipopolysaccharide (LPS) for activity (Moran et al. 1995; Valkonen et al. 1994, 1997), for example.

Iron-regulated protein

To overcome iron-limitation, *H. pylori* has developed several systems: extracellular siderophores (Illingworth et al. 1993) and lactoferrin-binding protein (Husson et al. 1993; Dhaenens et al. 1997). In addition, based on the complete genome sequence of *H. pylori* ATCC 26695, two orthologs of ferritins (proteins that store iron) have been found: Pfr (HO0653) and NapA (HO0243) (Frazier et al. 1993; Doig et al. 1993; Evans et al. 1995a).

Vacuolating cytotoxin and *cag* pathogenicity

The vacuolating cytotoxin (VacA; HP 0887 in *H. pylori* ATCC 26695) is a secreted and cleaved protein. Culture supernatants from Tox^+ *H. pylori* (active VacA) induce vacuolation in human primary gastric epithelial cells, in contrast with culture supernatants from an isogenic Tox^- strain (Smoot et al. 1996; Harris et al. 1996). The "cytotoxin-associated gene", *cag*A, was originally thought to be necessary for VacA vacuolating activity or expression due to the strong correlation in clinical isolates; *cag*A^+ strains were Tox^+ (type I) and *cag*A^- strains were Tox^- (type II) (Covacci et al. 1993; Tummuru et al. 1993; Xiang 1995). However, using an isogenic *cag*A mutant, it has been

shown that *cag*A is not required for expression or vacuolating activity of VacA (Xiang et al. 1995; Tummuru et al. 1994). Both type I and type II strains contain *vac*A sequences. Type I strains contain a 40 kb DNA fragment which is absent in type II strains (Censini et al. 1996). This fragment is called the *cag* pathogenicity island. Further findings indicate that type I (cag^+) strains are more virulent than type II (cag^-) strains, yet the presence of the *cag* pathogenicity island alone is not sufficient to confer full virulence.

Antibiotic Treatment of *H. pylori* infection

H. pylori seems fragile in an *in vitro* environment and its sensitivity to a large number of antibiotics, but only a few of antibiotics are active enough against the organism *in vivo* to cure the infection. Nitroimidazoles, for example, metronidazole and tinidazole, are metabolized in the bacterium to an active form that destroys the bacterial DNA. Macrolides, such as clarithromycin, erythromycin, roxithromycin, and azithromycin, block the RNA synthesis in the bacterium. Penicillins such as amoxicillin are active against the cell wall. And tetracycline inhibit protein synthesis. A proton pump inhibitor (H_2 receptor antagonist) such as omeprazole, inhibits gastric secretion, and facilitates the transportation of the drug to the target (Loo et al. 1992; Millar et al. 1992; Rubinstein et al. 1994).

The acidic gastric environment has been blamed for drug delivery deficiency. Therefore multitherapy has higher efficacy of antimicrobial activity *in vivo* than monotherapy. In dual therapy, amoxicillin, clarithromycin, azithromycin or tetracycline is given in combination with omeprazole. In bismuth-based triple therapy, bismuth is administered, plus a nitroimidazole combined with either tetracycline or amoxicillin.

Other triple therapy includes H_2 receptor antagonist, nitroimidazole and amoxicillin or tetracycline. Quadruple therapy includes omeprazole, bismuthdicitrate or bismuthsubsalicylate, nitroimidazole, and tetracycline or amoxicillin (Unge P. 1999).

The combination drug therapies have a synergistic antibacterial effect on eradicating *H. pylori*. Studies searching for even better eradication rates are ongoing and may modify treatment strategies in the future as new compounds and preventive modalities such as vaccines are developed.

Genetics and Metabolism of *Helicobacter pylori*

To date, the genomes of two *Helicobacter pylori* strains, ATCC 26695 and J99, have been sequenced. Tomb et al. (1997) identified 1,590 open reading frames (ORFs) representing 91% of the *H. pylori* chromosome of strain ATCC 26695. Among the 1,590 ORFs, 1,091 were found to have counterparts in other organisms allowing putative biological roles to be assigned. The 499 ORFs that exhibited no database matches are considered at this time to be specific to *H. pylori*.

The availability of the complete genome sequence of *Helicobacter pylori* ATCC 26695 has opened new avenues for research in the molecular biology of this pathogen (Taylor et al. 1999). Proteome analysis among the different *H. pylori* strains (Jungblut et al. 2000) has also provided a highly effective tool for the identification of new virulence or pathogenic factors and potential diagnostic tools. Based on comparison of the experimental data with those derived from the whole genome sequence, *H. pylori* has glucose metabolism, pyruvate metabolism and an incomplete Krebs cycle, amino acid,

fatty acid and phospholipid metabolism, which largely explain its complex nutritional requirements.

H. pylori and the acidic environment of the gastric mucosa

Upon entering the gastric lumen, *H. pylori* migrates from the low pH environment in the lumen to the less acidic mucus layer that covers the gastric epithelial cells. In the mucus layer there is a pH gradient from pH 2.0 at the luminal side to near neutral (pH 6-6.5) close to the epithelial cells (Schade et al. 1994). Approximately 10% of *H. pylori* cells are closely associated with the epithelial surface (Hessey et al. 1990). The majority of *H. pylori* live in the regions of the gastric mucus where the pH ranges from pH 4.5 to pH 5.5. Thus, *H. pylori* not only must survive the severe acid environment of the lumen in order to reach its niche, but also must grow at acidic pH, even though it is not an acidophile.

Occasionally, when the pH in the lumen drops below pH 1, the pH gradient in the mucus layer collapses, resulting in pH values as low as 2 close to the epithelial cell surface (Allen et al. 1997). Thus, *H. pylori* must withstand occasional acid shocks even after it has established itself in its niche. Several research investigators have reported that the acidic environment enhances the binding of *H. pylori* to human mucins (Veerman et al. 1997) and to human intestinal cell monolayers (Corthesy-Theulaz et al. 1996). These studies indicate that *H. pylori* is well adapted to living in the acidic mucus layer.

Acid resistance mechanisms in *H. pylori*

H. pylori optimal growth *in vitro* is between pH 6.0 and 7.0, with no growth at all at pH below 4.5 to 5.0 or above 8.0 (Morgan et al. 1987). However, it has developed

strategies to survive the extreme environment of the stomach. *H. pylori* produces copious amount of urease, which hydrolyzes gastric urea to ammonia (Owen et al. 1995). In the presence of urea, it can survive *in vitro* at pHs as low as 3.0 (Clyne et al. 1995). The ammonia can neutralize gastric acid and provide time to traverse the mucus layer and colonize the surface of the epithelium. Also, *H. pylori* utilizes exogenous urea as a nitrogen source for amino acid synthesis (Williams et al. 1996). The ability of *H. pylori* to survive exposure to low pH is likely to depend on a combination of cytoplasmic and surface-associated urease activities (Krishnamurthy et al. 1998). Low environmental pH reduces urease activity as well as synthesis of nascent urease, catalase, and presumably, many other proteins (Bouerfeind et al. 990). This suggests that *H. pylori* is not acidophilic, although it tolerates short-term exposure to low pH. The bacterium has adapted itself to the acidic environment of the stomach and can be classified as an acid-tolerant neutrophile.

Urease-negative *H. pylori* fails to colonize gnotobiotic piglets and this effect is dependent on an intact gastric pH (Eaton et al.1994). In fact, the acid environment of the stomach may be crucial for *H. pylori* survival in the presence of urea, *H. pylori* does not survive in the normal environment in the presence of urea because of the subsequent rise in pH rather than ammonia toxicity (Clyne et al. 1995).

In addition to the production of urease, *H. pylori* has developed other mechanisms of pH homeostasis. A few of these mechanisms are outlined below. The pH of a host microenvironment can be considered as one of the physicochemical signals that then results in the induction or repression of appropriate genes. McGowan et al. (1996, 1997)

reported a change in *H. pylori* protein content following a shift in extracellular pH, indicating the induction of special systems to allow acid tolerance.

In many bacteria, the H^+-translocating F_1F_0-ATPase is an important enzyme for regulating intracellular pH or synthesizing ATP. A transmembrane proton gradient or proton motive force (PMF) is formed by reversal of the F_1F_0-ATPase. In respiring cells, passage of electrons through an electron transfer chain to suitable electron acceptors is coupled to the extrusion of protons and the creation of a transmembrane electrochemical proton gradient (Kadner et al. 1996). The basic strategy of *H. pylori* is to maintain the PMF by adjusting the potential difference across the cytoplasmic membrane to compensate for the changes in pH gradient (Meyer-Rosberg et al. 1996, Sachs et al. 1996). This PMF is used to convert energy into ATP through the F_1F_0-ATPase; thus, the PMF may be kept at a high enough level to allow ATP synthesis over a wide range of external pHs.

Three proton-translocating P-type ATPases have been identified in *H. pylori*: ATPase-439 (CadA), ATPase-938 (CopA), and ATPase-115 (Leisinger et al. 1996, Maier et al. 1996, Manos et al. 1998). Tomb et al. (1997) suggested that they are more closely related to divalent cation transporters. And are involved in importing divalent cations and eliminating toxic metals rather than in pH regulation. However, HP1522 and HP1183 are similar to genes coding for the Na^+/H^+ antiporters in *E. coli* and *Enterococcus hirae*, respectively, where these H^+-coupled ion transport systems are responsible for controlling the flow of ions into and out of the cell and play a role in pH regulation.

Basic amines, products of amino acid decarboxylases, provide an important protective mechanism for bacteria living under low-pH conditions, and may be involved

in pH homeostasis in *H. pylori*. The HP0422 gene is similar to *speA*, encoding arginine decarboxylase in *E. coli*. This enzyme may play a role in regulating pH by utilizing products of protein biodegradation (Olson et al. 1993).

H. pylori could create a positive-inside membrane potential by either concentrating cations inside the cells or pumping anions out of cells. Tomb et al. (1997) suggested that the first strategy is more likely since no clear mechanism for anion efflux has been identified. To date, genes coding for only three anion transporters have been found: HP0473 to HP0475, encoding the ABC transporter ModABC involved in the uptake of molybdenum; HP0313, encoding the nitrite permease; and HP1491, encoding the phosphate permease.

H. pylori persists in the human stomach where it may encounter a variety of DNA-damaging conditions, including gastric acidity. Thompson et al (1998) research data suggested that *H. pylori* nucleotide excision repair (NER) pathway is involved in the repair of acid-induced DNA damage, in addition to acid resistance mechanisms listed above.

Phosphate uptake regulation in *H. pylori*

Orthophosphate plays a pivotal role in cell functioning. It is involved in most metabolic energy transductions and serves as an intermediate in the biosynthesis of numerous metabolites.

In terms of cellular content, phosphorus is the third most abundant element (behind carbon and nitrogen). Glucose- or acetate-grown *E. coli* cells contain about 15 mg of phosphate per gram (dry weight) (Damoglou, 1968). Phosphate compounds serve

as major building blocks of innumerable biomolecules; phosphate is an essential component of membrane lipids, complex carbohydrates such as lipopolysaccharides, and nucleic acids, and phosphate is incorporated into many proteins posttranslationally. Phosphate compounds are also especially important in energy metabolism because of the biological role of high-energy phosphanhydride bonds. Therefore, phosphate metabolism involves numerous metabolic pathways, in addition to those that may have a specific role in the process of assimilating phosphorous from the environment. Some pathways of phosphate metabolism may be of primary importance for phosphate assimilation during exponential growth, while others may be important for scavenging phosphate compounds from the environment for survival under conditions of phosphate limitation, or for storage of high-energy phosphate compounds under conditions of excess carbon and energy or limitation biosynthetic capacity.

Inorganic phosphate exists in three chemical forms that are of biological importance; inorganic phosphate (P_i), pyrophosphate (PP_i), and metaphosphate (polyphosphate [poly (P_i)], also called volutin). Of these, only P_i appears to be taken up from the environment.

The phosphate uptake mechanism in *Saccharomyces cerevisiae*

The phosphate transporter process in *S. cerevisiae* is characterized by a high-affinity transport system operative at low (μM) concentrations of phosphate and a low-affinity transport system operative at high concentrations (mM) of phosphate (Persson, 1998). Previous studies of the high-affinity system in intact cells of *S. cerevisiae* have revealed that the kinetic parameters K_m and V_{max} are dependent upon the prevailing

cellular ion concentration and pH. The low-affinity system is a constitutively expressed P_i/H^+ co-transporter with optimum of pH 4.5 (Pattison-Granberg, et al. 2000). In contrast, the high-affinity system (K_m= 1 to 15 μM) is derepressible by phosphate starvation during aerobic and anaerobic cell growth with optimum of pH 7.0. Of the proteins responsible for transport of phosphate into the cells, PHO-84 is a P_i/H^+ co-transporter with an optimum pH 4.5 for phosphate uptake similar to that of the constitutive low-affinity system (Martinez et al. 1998b). The other PHO-89 is a P_i/Na^+ co-transporter with an alkaline pH optimum, largely inactive at pH 4.5 (Martinez et al. 1998a). The phosphate transport systems of *Neurospora crassa* are similar to those characterized in *Saccharomyces cerevisiae*, except that the permeases are affected by high pH (above 7.0) instead of low pH.

The phosphate uptake mechanism in *E. coli*

E. coli uses two kinds of compounds as primary phosphate sources: (i) inorganic phosphates, (ii) organophosphates (phosphate esters). Regardless of the phosphate source, the overall process of phosphate assimilation has at least two common steps. The first step involves uptake of the phosphate compound, few organophosphates and inorganic phosphates are taken up intact directly; most organophosphates are hydrolyzed in the periplasm, the P_i released and then taken up by the phosphate transporter. A later step involves entry of P_i or a phosphoryl group of an alternative phosphate compound into ATP (or a metabolizable organophosphate) via one of several steps in central metabolism.

There are two phosphate transport systems present in *E. coli*, the phosphate inorganic transporter (Pit) and the phosphate-specific transporter (Pst) (Rosenbery et al. 1977). The Pit system appears to be a proton/phosphate symporter, whereas the Pst system is a typical periplasmic permease that comprises one periplasmic substrate-binding protein; and three membrane-bound components (Ames, 1998), and belongs to the large family of ABC transporters (Hyde et al. 1990, Ames and Joshi, 1990). The Pit system is made constitutively and has a K_m for phosphate of about 25 μM (Rosenberg et al. 1977). The Pst system has a higher affinity for phosphate with a K_m of about 0.2 μM (Rosenberg et al. 1977). The synthesis of the Pst system is regulated by the concentration of phosphate in the growth medium and has the somewhat unusual property of being a sensory adjunct to a two-component (PhoR, PhoB) regulatory system that controls the promoters of the Pho regulon (Wanner, 1993). However, the PhoR sensor has, comparatively, a much-reduced periplasmic domain (Scholten and Tommassen, 1993) and it is the Pst system that appears to carry out the sensory role. Experiments with mutations of the *pst* operon established that the two functions, P_i transport and Pho regulation, are independent. In fact, many of the *pst* mutants are insensitive to repressible amounts of P_i in the medium. As mentioned before, the genomic sequences of two strains of *H. pylori* (ATCC 26695 and J99) have been published. From these sequences, transport and binding protein coding regions, HP1491 from ATCC 26695, and HP1384 from J99, were each shown to have 533 amino acids and were 97.4% identical. Based on the comparison of clusters of orthologous groups of proteins (COGs) both were shown to have significant similarity to the phosphate permease of *Haemophilus influenzae* (PH0640), *Saccharomyces cerevisiae* (YBR296c), and *E. coli* (PitA, PitB).

In addition to HP1491 and HP1384, several open reading frames in the sequenced genomes encode putative proteins belonging to the ABC (ATP-binding cassette) transporters and could potentially be involved in the transport of phosphate into *H. pylori* under various environmental conditions.

Summary

In the following work, pH effect on orthophosphate uptake and incorporation in *H. pylori*, plus cloning, expression and partial characterization of the phosphate permease (HP1491) is presented. The investigation addresses the physiological regulation of P_i transport, especially during environmental changes in pH and phosphate concentration, and the comparison of phosphate uptake between wild type *H. pylori* (ATCC 26695) and a mutant strain (HPmt1491, phosphate permease was disrupted). The work presented attempts to answer the following: Does the phosphate permease encoded by HP1491 from ATCC 26695 play a major role in P_i uptake in *H. pylori*? Do phosphate concentration and pH as observed in *Saccharomyces cerevisiae* and *Neurospora crassa* affect phosphate uptake in *H. pylori*?

Chapter Two

MATERIALS AND METHODS

Cultures and Growth media

- **Strains and plasmids**

Helicobacter pylori strain ATCC 26695 was purchased from American type Culture Collection (ATCC) (Rockville, MD, USA). *Escherichia coli* strain ER1793 (New England Biolabs) was used as the host for all plasmid preparations. The *H. pylori* expression vector pBCα3 was a gift from J. J. E. Bijlsma (Vrijie Universiteit Amsterdam, The Netherlands).

- **Bacterial cultures**

Helicobacter pylori ATCC 26695 was used as the parental strain in this study. Cells were routinely cultured on solid brucella agar with 5% lysed sheep blood and 10 μg/ml nalidixic acid, under microaerophilic condition (5% O_2, 10% CO_2, 85% N_2) using a CampyPak for 48 hours at 37°C.

One blood agar plate was scraped into modified Tryptic Soy broth. Modified Tryptic Soy broth (mTSB) is composed of pancreatic digest of casein (17.0g/L); papaic digest of soybean meal (3.0g/L); NaCl (5.0g/L); dipotassium phosphate (10 μM, 50μM, 14mM); serum (10%), glucose (0.2%). Liquid cultures were grown for 24 hours at 37°C with shaking (200rpm) under microaerophilic conditions. *H. pylori*

culture was stored in brucella broth with 15% glycerol, 10% (vol/vol) serum at –70°C.

Quantitation of bacteria was achieved by optical density measurements at 600nm and compared to previously performed growth curves and viability.

- **Plasmids**

E. coli ER1793 was used as a host for recombinant plasmids. ER1793 was routinely cultured in either liquid or solid Luria-Bertani (LB) medium. For selection, ER1793 media were supplemented with 50mg/L kanamycin or 20mg/L ampicillin. Recombinant plasmids were transformed and maintained in *E. coli* ER1793.

- **Low and high phosphate media**

Modified TSB without dipotassium phosphate was called TSB^{op}. Low phosphate media (LP-mTSB) contained 10μM dipotassium phosphate; high phosphate media contained 14mM dipotassium phosphate.

- **pH media**

Tris/Succinate (25mM) was used to adjust modified Trypticase Soy broth to different pHs.

- **Buffers**

SDS/electrophoresis buffer, 5x

Tris base (15.1g), 72.0g glycine, 5.0g SDS are mixed in deionized H_2O (dH_2O) sufficient to make 1 liter. It is not necessary to adjust the pH of the stock solution. When the solution is diluted for use, the pH will be 8.3.

SDS/sample buffer, 2x

To 40ml of dH_2O: 1.52g Tris base, 20ml glycerol, 2.0g SDS, 1mg Bromphenol Blue are added and the pH adjusted to 6.8 with 1N HCl. The volume is then brought to 100ml with dH_2O.

TE buffer, pH 7.5

TE buffer consists of 10mM Tris-Cl plus 1mM EDTA and the pH is adjusted to 7.5 for use.

Tris/Succinate, 1 M

Dissolve 121g Tris base in 800ml dH_2O, adjust to desired pH with concentrated succinic acid, mix and add dH_2O to 1 liter.

Labeling assay

- ***In vivo* [^{32}P]-orthophosphate labeling.** Modified TSB grown cells were harvested, washed three times with LP-mTSB, and preincubated one hour in LP-mTSB to allow the cells to adapt to low phosphate. The cells were then harvested and resuspended in fresh LP-mTSB at various pHs (pH 2-7) plus 20 μCi [^{32}P]-orthophosphate using the labeling protocol from Dannelly et al. (1989). After 8 hours incubation, 5μM urea was added to representative cells from each pH. Cells were allowed to incubate one hour, harvested, and washed three times in 50mM Tris-HCl, pH 7.5. Pellets were resuspended in 50mM Tris-HCl (pH 7.5) and aliquots removed for protein determination, liquid scintillation counting, and SDS-PAGE.
- ***In vivo* [^{35}S]-methionine labeling.** Cells were grown in mTSB for 24 hours, washed two times with L-methionine-free Minimal Eagle Medium (MEME), and

resuspended in a volume of MEME necessary to match optical densities of approximately 2.5 OD_{600} units. Cultures were pre-incubated for 90 minutes in MEME, at which time 65 μCi of [^{35}S]-methionine were added, as modified from Mizoguchi et al (1998). Labeling was stopped after 30 min by addition of unlabelled L-methionine to a final concentration of 8 mg/ml. Bacteria were harvested, washed, resuspended in 50 mM Tris-HCl, pH 7.5, and aliquots removed for protein determination, liquid scintillation counting, and SDS-PAGE.

- **Protein determinations** were made using the method of Bradford (1976). Aliquots of cell suspensions were mixed in an equal volume of 2% SDS sample treatment buffer and heated to 90°C for 5-8 minutes to rupture cells and denature proteins. Protein (0.03mg, 0.1mg) was loaded into each well of the 10% SDS polyacrylamide gel (SDS-PAGE) and electrophoresed (Laemmli, 1970).

  Aliquots of labeled cells were mixed with non-toluene based scintillation fluid and counted using a Packard LS5800 Series Liquid Scintillation System.

DNA manipulation

- **Enzymes, primers, and cloning**

  DNA restriction and modification enzymes used in the cloning experiments were obtained from Promega (WI, USA). Integrated DNA Technologies, INC (Coralville, IA, USA) produced oligonucleotides used as primers. Preparation and cloning of DNA fragments and vectors was performed by standard techniques (Ausubel et al. 1994).

- **Genomic DNA preparation**

The preparation of genomic DNA from *Helicobacter pylori* was performed using a modification of the method proposed by Owen and Bickley (1997). Briefly, heavy growth *H. pylori* cells from a blood agar plate were resuspended in 1ml 1x TE buffer to $1x10^8$ CFU/ml. The cells were collected by centrifugation and the supernatant removed. The bacterial pellet was resuspended in 400µl of lysis buffer A (2mM Tris-HCl, pH 8.0, 25mM glucose, 1mM EDTA, 100µg/ml lysozyme). The mixture was incubated for 30 minutes at 37°C. Then 300µl of lysis buffer B (50mM NaCl, 1% (w/v) SDS, 2.5mg/ml proteinase K) was added to the mixture. After 1-hour incubation at 37°C, phenol/chloroform extraction was performed and sample was centrifuged at 16,000 x g for 10 minutes and the aqueous phase containing the DNA was collected and concentrated by ethanol precipitation.

- **Construction of the Suicide Vectors**

Constructing pBCα5, (pBCα3 with 550 bp of HP1491 gene)

Genomic DNA was isolated from *H. pylori* and used as a template to amplify the 550 bp fragment of HP1491 gene by polymerase chain reaction (PCR) (Ausubel et al. 1994). The amino terminal primer was: 5'-ACA CGG ATC CTC TTA AGC GCT CCC TTA AAA AG-3' (corresponding to the c-terminal nucleotide sequence of the antisense strand of HP1491 from +1226 to +1257), with the additional 6-bp underlined sequence encoding the *Bam*HI restriction site and ACAC at the 5' end. The carboxyl terminal primer was: 5'-ACA CGG ATC CCG CAA TTA GAA AAC

AGC CAT GA-3' (corresponding to HP1491 nucleotides from +830 to +851), with a 6-bp *Bam*HI restriction site (underlined) and ACAC at the 5' end. The resulting amplification product was digested with *Bam*HI and cloned into pBCα3 also digested with *Bam*HI. This vector was designated pBCα5 and transformed into ER1793 by electroporation (Ausubel et al. 1994).

- **Natural transformation of *H. pylori***

H. pylori was naturally transformed essentially as described in Wang (1993), according to the following protocol. A heavy loop of cells (2 –3 days old) from blood agar was inoculated in quarter-size patches (8-10mm diameter) on a new blood agar plate and grown for 8 hours under microaerophilic conditions. Subsequently, 15μl of TE, containing approximately 2μg target plasmid, was added to each patch. After 16-24 hours incubation, the bacteria were harvested and resuspended in 1ml of brucella broth. The bacteria were pelleted, resuspended in 100μl of brucella broth, and plated on 20mg/L kanamycin selective plates. After 3-4 days incubation, single colonies were picked and grown on blood plates or brucella broth supplemented with 10% newborn calf serum.

Phosphate transport assay

H. pylori cells grown to stationary phase (OD_{600}=0.4) were washed once in a phosphate-free medium (TSB^{op}) followed by the addition of 10μl of [^{32}P]-orthophosphate

(3.5 x10^4 cpm/μmol) to 30 μl aliquots of cells, suspended at an OD_{600}=10 in 25 mM Tris-Succinate pH 7.2 to a final concentration of 50 μM P_i.

The suspension was immediately shaken well and incubated at 25°C. Phosphate transport assays were terminated at given time (2, 4, 6, 8, 10, 20 minute) by quenching of the reaction with 1 ml ice-cold Tris-Succinate dilution buffer. The sample was filtered (Millipore 0.2 μm GTTP) immediately and washed once with the same cold solution. Radioactivity retained on the filters was determined by liquid scintillation spectrometry. The unit of phosphate transport rate was determined as pmole/mg protein.

Chapter Three

Effect of pH on Orthophosphate Uptake and Incorporation in *Helicobacter pylori*

Summary

Protein phosphorylation occurs in prokaryotes in response to changes in environment, such as: mechanisms of modulation of enzyme activity, signal transduction, as in two component regulatory systems, and global control or crosstalk mechanisms. The purpose of this study was to determine if similar phosphorylation mechanisms might occur in *Helicobacter pylori*.

Helicobacter pylori (ATCC 26695) was grown to mid-logarithmic phase, and transferred to fresh glucose medium at pH 4.5, with or without 5μM urea, plus 20 μCi [^{32}P]-orthophosphate and incubated for 8 hrs. Labeled cells were lysed and electrophoresed on SDS-PAGE. Autoradiography showed numerous phosphorylated bands in control cells. However, pH 4.5 medium significantly reduced the uptake and incorporation of [^{32}P]-orthophosphate. On the other hand, cells grown at pH 4.5 plus urea significantly increased [^{32}P]-orthophosphate uptake and incorporation, unlike neutral pH plus urea, showed no significant differences from control cells. Total cell counts in pH 4.5 media were found to be lower than control cultures at 8 hrs, indicating a longer lag period than normal, but cells increased to normal population density by 24 hrs. No significant differences in cell number from other media were observed. The working pH of the four cultures was monitored throughout the experiment; at 8 hrs, low pH cultures,

with and without urea, remained at 4.5; however, at 24 hrs, the pH of both had increased to 6.0.

Objective

Survival of *H. pylori* in an acidic environment has been attributed to the presence of urease activity, which liberated ammonia from urea. One recent paper concluded that external pH induced regulation of intracellular urease activity, increased periplasmic pH and membrane potential, and stimulated protein synthesis (Mizoguchi et al. 1998). The purpose of this study was to observe the effects of changes in pH of surrounding media phosphate uptake and incorporation and on protein phosphorylation patterns in *Helicobacter pylori.*

Material and Methods

Culture conditions: Cells were cultured on blood agar plated containing 5% (vol/vol) alsevers sheep blood, and 10ug/ml nalidixic acid for 2 days at 37°C under microaerophilic conditions. Bacteria were confirmed as *H. pylori* on the basis of colony morphology, Gram reaction, and the production of oxidase and catalase. Strains were stored in brucella broth containing 15% glycerol, 10% (vol/vol) serum at –70°C. Bacteria were cultured in the mTSB by scraping the growth from one plate to inoculate the broth and cultures were grown for 24 hours at 37°C with shaking (200 rpm) under microaerophilic conditions ($OD_{600nm} = 0.8$).

Electrophoresis: Cells were resuspended in 40 µl of 50mM Tris-HCl buffer with 2% SDS treatment buffer, and the mixtures are heated to 90°C for 5-8 minutes. Protein (30

μg, 0.1mg) was loaded in each well of a 10% SDS-PAGE as described previously (Laemmli, 1970).

Results

Figure 3.1 showed no significant difference in growth rate of control cells (blue) or cells in neutral media plus urea (green) for 36 hours. However the rate of growth of the acid cells was significantly reduced, while the rate of growth of the cells in acidic condition with 5 μM urea present was higher than control cells throughout the 48 hours trial period. Monitored pH values from growth curves and were plotted in figure 3.2. Control cells maintained a relatively constant pH for 24 hours followed by a slight elevation to pH 7.7 at 36 hours till 48 hours. Neutral cells plus 5 μM urea increased to pH 7.7 at the first measurement (4 hours) and remained at 7.5 or above for the remainder of the trial. The cells in acid medium slowly increased from pH 5.2 to pH 6.3 at 48 hours. Cells in acid medium plus 5 μM urea adjusted the pH from 5.2 to 6.7 within the first 4 hours and the pH remained fairly constant through 48 hours.

Cultures were incubated for 8 hours in the presence of [^{32}P]-orthophosphate at neutral pH, acidic pH, and acid pH plus urea with glucose as the sole source of carbon. The cells were harvested, lysed and loaded on SDS-PAGE and electrophoresed. The autoradiograph from SDS-PAGE was shown in figure 3.3. The gel was over exposed therefore distinct bands were visible on the film. Neutral cells were shown in lane 1, acidic in lane 2, and acidic plus urea in lane 3. The pattern observed due to phosphate incorporation into protein changed dramatically in the acid-grown cells; the heaviest

labeled band at approximately 30 kdal was no longer labeled but reappeared when urea was present, returning the cells to a more neutral pH. The labeled band at approximately 36 kdal appeared more distinctly in the acid grown cells than in neutral pH. However, most interesting, the total amount of label in the acid grown cells was greatly reduced when compared to either lane 1 or 3 indicating that total cellular uptake of phosphate was reduced at low pH.

When cells were grown at various pHs, with and without urea, it was observed that phosphate uptake was dramatically reduced at pH 4.0 and below and the addition of urea to the medium did not allow recovery of uptake (figure 3.4). At pH 4.5 and 5.0, phosphate uptake was significantly reduced 86% and 15%, respectively, as compared to pH 7.0, but the addition of urea to the medium allowed complete recovery of phosphate uptake.

Protein synthesis was monitored in acidic grown cells by [^{35}S]-methionine labeling. As seen in figure 3.5, autoradiography of SDS-PAGE showed that cell extracts from neutral cells (lane 1), pH 4.5 cells (lane 4) and pH 4.5 plus urea (lane 5) had equivalent [^{35}S]-methionine incorporation. However, at pH 3.0 (lane 2), whether urea was added (lane 3) or not, the [^{35}S]-methionine incorporation was significantly reduced indicating that protein synthesis was shutting down and rapid recovery of pH (with urea cells) did not reverse the situation.

Total cell [^{35}S]-methionine uptake was compared by counting the well washed, labeled cells after washing extensively (figure 3.6). Uptake was observed to be significantly reduced in low pH cells (3.0, 4.0, and 5.0) and was incompletely recovered when pH was recovered, by addition of urea to the medium. However, the reduction in

uptake was not responsible for reduction of incorporation since uptake was significantly reduced at pH 4.5 and 5.0 but incorporation was not.

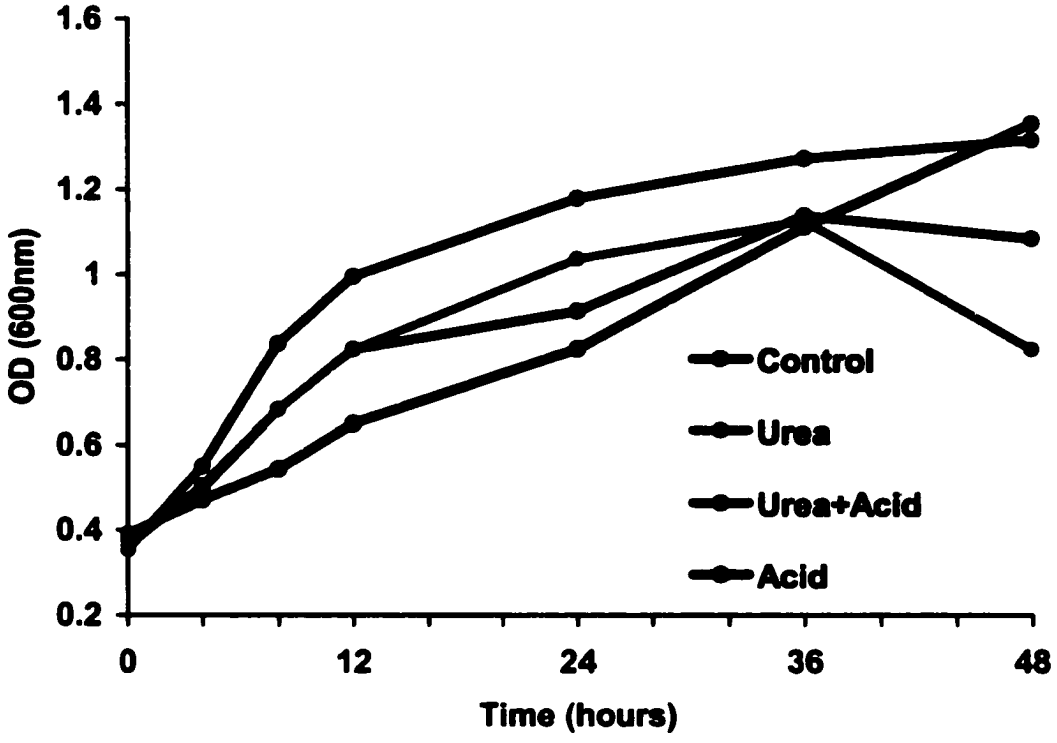


Figure 3.1 Growth curve of *H. pylori* under different pH media. Glucose-grown cells (blue line, as control), glucose with 5μM urea cells (green line), glucose with 5μM urea under acidic cells (red line) and glucose without urea under acidic cells (brown line) were grown on modified Tryptic soy broth, at different time point, samples were collected and OD were measured at 600nm. Points are the average of three independent experiments, S.E.

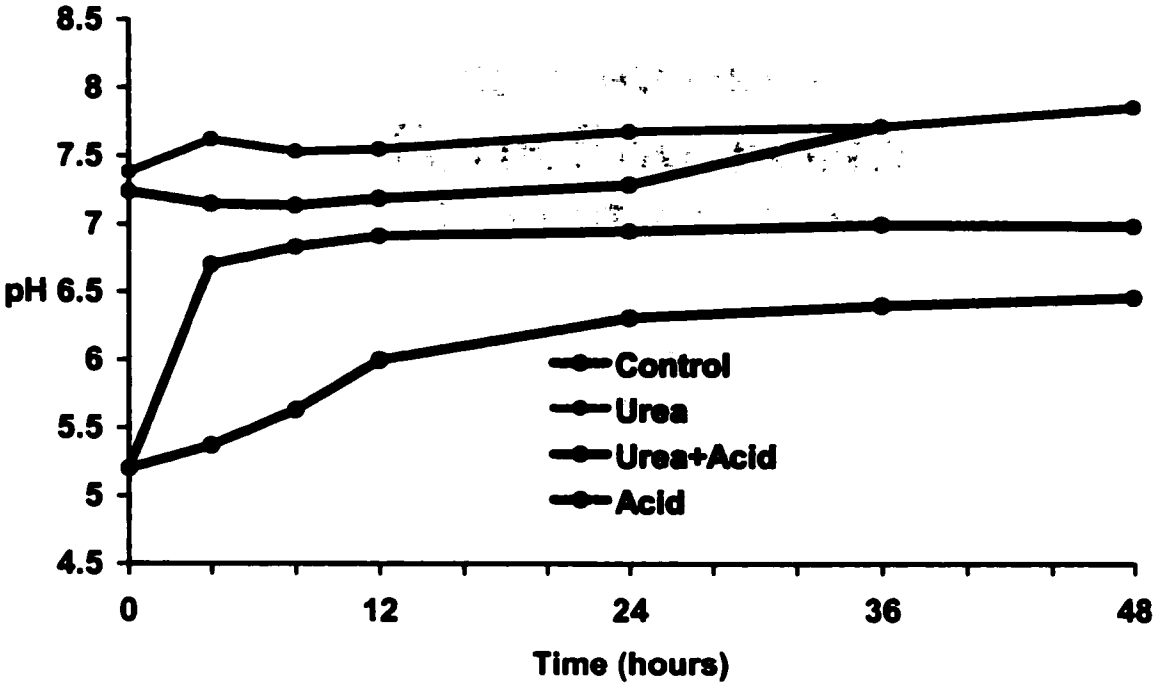


Figure 3.2. pH curve of *H. pylori* under different pH media. Glucose-grown cells (blue line, as control), glucose with 5μM urea cells (green line), glucose with 5μM urea under acidic cells (red line) and glucose without urea under acidic cells (brown line) were grown on modified Tryptic soy broth, at different time point, samples were collected and pH were measured. Points are the average of three independent experiments, S.E.

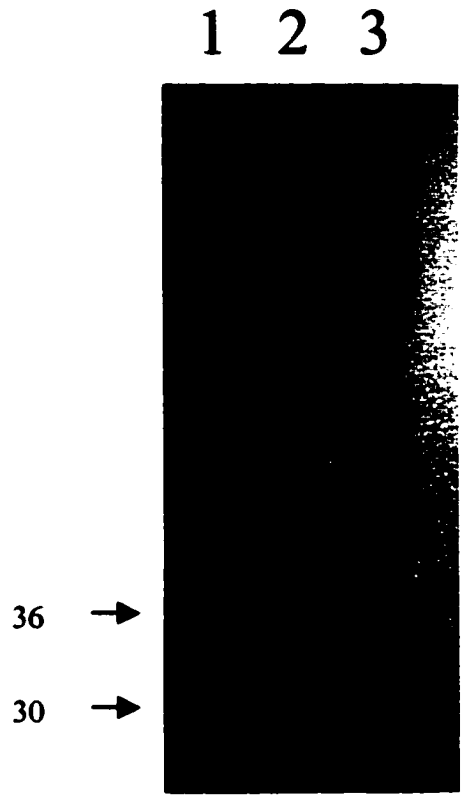


Figure 3.3. Autoradiography of [^{32}P]-orthophosphate labeled cell extracts. Glucose grown cells (lane 1), glucose grown cells under acidic medium pH 4.5 (lane 2) and glucose grown cells with 5 μM urea under acidic medium pH 4.5 (lane 3). 30μg protein were loaded on each lane.

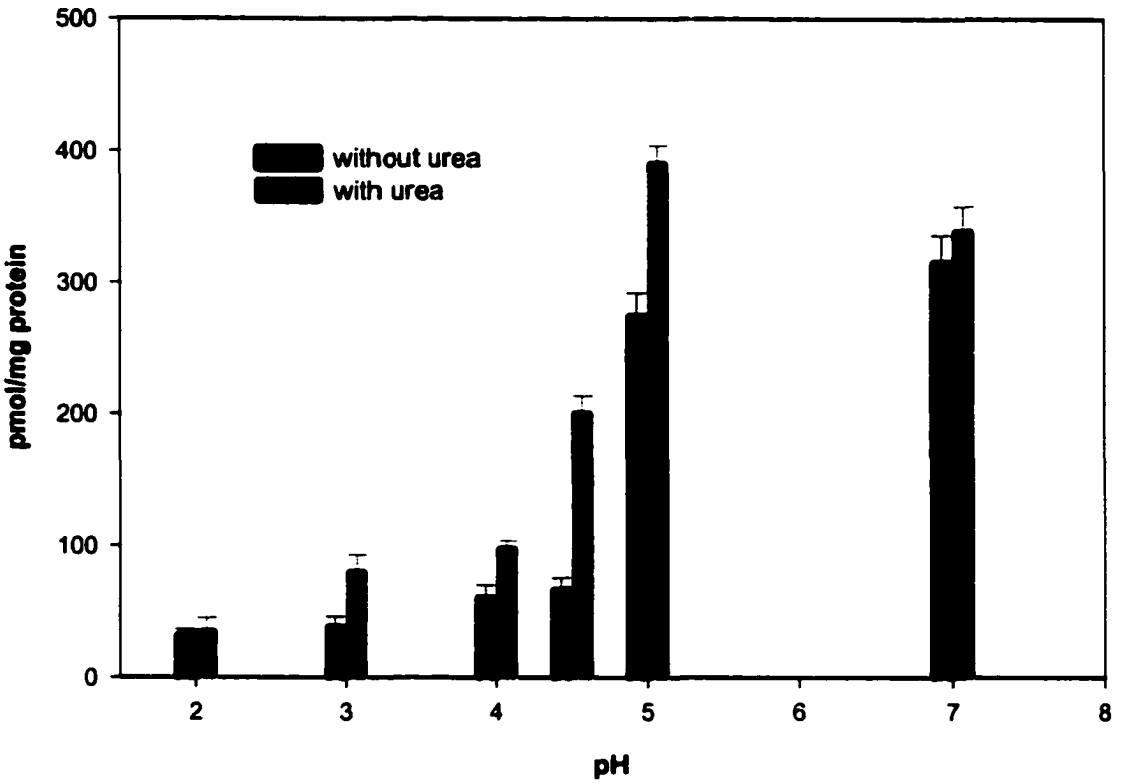


Figure 3.4. pH effect on phosphate uptake in ATCC 26695. Cells were grown in modified Triptic soy broth at different pH medium with (light blue bar) or without (dark blue bar) 5μM urea. Phosphate uptake activities were assayed as described in materials and methods.

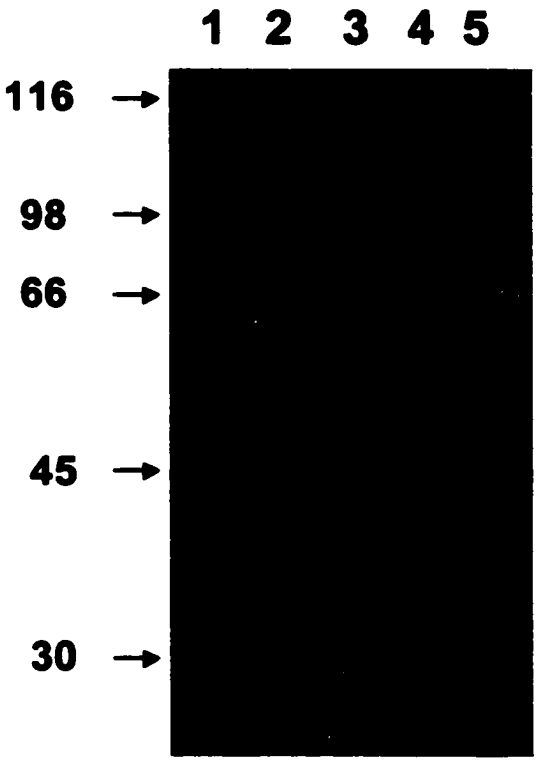


Figure 3.5. Autoradiography of [^{35}S]-methionine labeled cell extracts. Glucose grown cells at pH 7.0 medium (lane 1), glucose grown cells at pH 3.0 medium (lane 2), glucose grown cells at pH 3.0 with 5μM urea (lane 3), glucose grown cells at pH 4.5 medium (lane 4) and glucose grown cells with 5μM urea at pH 4.5 medium (lane 3). 0.1mg protein were loaded on each lane.

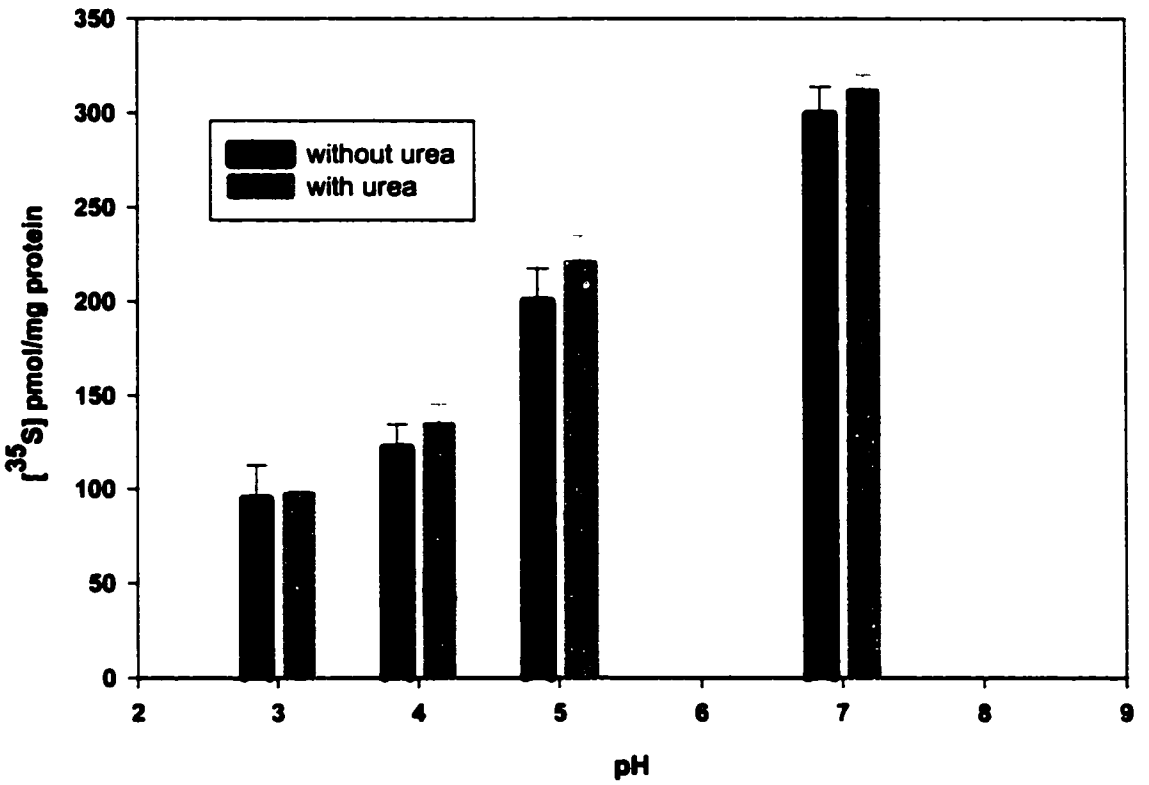


Figure 3.6. pH effect on [^{35}S]-methionine uptake in ATCC 26695. Cells were grown in modified Triptic soy broth for 24 hours, then cells were harvested and washed two times in L-mehionine free medium MEME, cells were resuspended (OD_{600}=2.5) in different pH MEME media, after 90 minutes preoccupation, incubations were continued after addition of 65 μCi of [^{35}S]-methionine. After incubation for 30 minutes, labeling was stopped by addition of unlabelled L-methionine to a final concentration of 8 mg/ml. Cells were harvested and washed as described in materials and methods.

Discussion

Growth studies revealed that the pH of acidic media could be recovered within 4 hours when urea was added to the medium and the return to neutrality significantly increased the growth rate. Comparison of growth curves at pH 7.2 and urea-recovered cells at pH 6.7 indicated that either cells grew optimally at pH 6.7 and the rate was reduced at pH 7.2 or the presence of urea caused an increase in growth rate. The same increase was not observed, however, when urea was added to pH 7.2 cells, increasing the pH to 7.7. In favor of the hypothesis that urea boosted initial growth rates was the observation that urea caused a change in the rate of protein synthesis (Mizoguchi et al.1998). However, in studies addressing the effect of ammonia on the viability of *H. pylori* (Neithercut et al. 1991), it was observed that high concentrations of urea (1000 x times greater than used in this study) killed cells because the ammonia concentration produced by the urease was greater than the concentration of ammonia that can be pumped out of the cell.

Also, from the growth studies, it was observed that *H. pylori* in an acidic environment without urea could slowly alter the pH of the external environment, returning it to near neutral after approximately 12 hours. The effect on growth was to produce steady growth over the 48 hours trial unlike the normal biphasic curve. This observation has also been made in other Gram negatives, such as *E. coli* (Damoglou et al. 1968).

Observation of changes in phosphate uptake and incorporation under acidic conditions (figure 3.3) revealed that there are major changes in both uptake and

incorporation. However both appeared to be reversible upon restoration of the external pH, provided the acid medium which did not drop below pH 4.5.

Experiments also showed that the changes in phosphate uptake and incorporation were not due to significant alterations in protein synthesis, while changes occurred in [^{35}S]-methionine uptake, incorporation, and therefore the rate of protein synthesis did not appear to change. Mechanisms for pH change and changes in uptake and incorporation of orthophosphate are under investigation.

Chapter Four

Generation of phosphate permease mutant (HPmt1491) and characterization of phosphate uptake compared to wild type (ATCC 26695)

Summary

Phosphate is an essential nutrient for all cells and its availability in the environment varies widely. We have observed, using [^{32}Pi]-incorporation to determine changes, that phosphate transport was proportionate to phosphate concentrations in the media. Also, we showed previously that the rate of uptake was dependent upon the pH of the medium; phosphate uptake was greatly reduced in acidic environments. Our results indicated that *H. pylori* may possess a unique phosphate regulatory mechanism to accommodate changes in pH and phosphate availability that may be important in determining where the organism can survive. Publication of genomic sequences of two strains of *H. pylori* revealed genes with significant similarity (59.6%) to the phosphate permease of *Haemophilus influenzae*. In addition, these genes show strong similarity to phosphate permeases/ion co- transporters from *Neurospora crassa* and *Saccharomyces cerevisiae*, which also are regulated by phosphate limitation and pH. The genes, HP1491 from ATCC 26695 and HP1384 from strain J99, code for proteins with 420 amino acids. We have put HP1491 gene into suicide plasmid pBCα3 to allow partial characterization of its activity, especially during changes in pH and phosphate concentration. We found that the activity of phosphate uptake were similar to Pho89 in *S. cerevisiae*.

Objectives

H. pylori is distinguishable from other organisms on the basis of its acid resistance. Urease analysis and studies have provided an increased understanding of this characteristic. However, few of *H. pylori* transporters mediating the uptake of nutrients have been characterized at the genetic, molecular or biochemical levels. There are no studies of the role of nutrient transport, such as the nature and availability of carbon, nitrogen, or energy sources in the acidic survival of *H. pylori.* Transport of phosphate across the plasma membrane is the first obligatory step of phosphate utilization in the cells. Several studies demonstrated that phosphate and/or phosphorylation has major effects on the survival of soil and medical important organisms, such as, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae, Streptomyces griseus,* etc.

Because of its importance as a human pathogen, we are interested in the physiology and metabolism of nutrient uptake systems, especially of phosphate uptake system. Although the kinetics of the phosphate uptake of *H. pylori* could be measured in cellular biochemical level, the molecular basis of these transport processes have remained obscure until the recent publication of the genomic sequences of two strains *H. pylori* ATCC 26695 and J99. The complete genomic sequence of *H. pylori* ATCC 26695 revealed the gene HP1491, a potential phosphate permease in *H. pylori*, with significant homology to the phosphate permease transporter from *Saccharomyces cerevisiae* (as discussed in chapter one).

The aim of this study is to examine the role of phosphate permease encoded by HP1491in phosphate transport during changes in pH and phosphate concentration.

Materials and Methods

Bacterial strains, plasmids, culture conditions. *H. pylori* ATCC 26695 was used as the parental strain in this study and *E. coli* ER1793 was used as a host for recombinant plasmids. pBCa3 is a *H. pylori* integration, suicide plasmid, HP1491 gene fragment was amplified by PCR using primers with engineered *Bam*HI restriction sites at both ends. The HP1491 gene fragment was ligated into the dephosphorylated unique *Bam*HI site of pBCa3 and transformed into *E. coli* ER1793. The recombinant plasmid pBCa5 was amplified and isolated from transformed *E. coli* on LB media with kanamycin. Finally pBCa5, which carries the gene of HP1491 insert, were naturally transformed into *H. pylori* and HP1491 mutants (HPmt1491) were then screened on blood plate with kanamycin. All standard DNA manipulations, to include electroporation and transformation procedures were performed as described by Sambrook et al (1989). Biochemical verifications of phosphate uptake were performed by assaying intracellular [γ-^{32}P]-orthophosphate uptake between the wild type (ATCC 26695) and mutant (HPmt1491) stains under defined conditions. Procedures were performed as described by Cox et al (1981).

PCR primer design. The left primer sequence used was: 5' ACA CGG ATC CCG CAA TTA GAA AAC AGC CAT GA 3', corresponding to the HP1491 nucleotides from +830 to +851, with a 6-bp *Bam*HI restriction site and ACAC at the 5' end. The right primer sequence used was: 5' ACA CGG ATC CTC TTA AGC GCT CCC TTA AAA AG 3', corresponding to the C-terminal nucleotide sequence of the antisense strand of HP1491

from +1226 to +1257, with an additional 6-bp sequence encoding the *Bam*HI restriction site and ACAC at the 5' end.

Phosphate transport assay. P_i uptake by the cells of ATCC 26695 and mutant cells of HPmt1491. The cells were shaken at 37°C in low or high P_i medium. The cells were collected when cell growth reached an optical density at 600nm of 1.0, washed, and inoculated into low-P_i medium to give a cell concentration with an optical density at 600nm of 0.5. The radioactivity of the medium was adjusted to 2nmol/μl with [^{32}P]-orthophosphate. The amount of P_i absorbed by the cells was expressed as counts per minute of [^{32}P]-orthophosphate radioactivity per mg protein. Transport assays were terminated at different time points by adding ice-cold buffer. The sample was filtered and the filter washed with cold buffer. Radioactivity retained on the filters was determined by liquid scintillation spectrometry.

Sequence alignment

Consensus key (see documentation for details)
* - single, fully conserved residue
: - conservation of strong groups
. - conservation of weak groups
  - no consensus

CLUSTAL W (1.8) multiple sequence alignment

```
Y64o_       ------------------------------------MEIISQYGSWLV
Pho4_       -------------------------------------MLPDPTVLL
PHO89_      -------------------------------------MALHQFDYIF
PitB_       ----------------------------------MLNLFVGLDIYTGLLL
PitA_       ----------------------------------MLHLFAGLDLHTGLLL
HP1491_     MEIKNIKEFEKASKKLQKDTLKIALALLFLIGAALLALIFGQANSKGLLL

:.

Y64o_       WITAVFGFFMAFGIGANDVSNSMGTSVGSGTITAKQAIIIALIFESAGAY
Pho4_       IIGAVIAFILAFAIGANDTANSFGTSVGSKVLTLHQAYVLASIFETLGAC
PHO89_      AIAMLFAFLDAFNIGANDVANSFASSISSRSLKYWQAMVLAGLCEFLGAV
PitB_       LLALAFVLFYEAINGFHDTANAVAAVIYTRAMQPQLAVVMAAFFNFFGVL
PitA_       LLALAFVLFYEAINGFHDTANAVATVIYTRAMRSQLAVVMAAVFNFLGVL
HP1491      IFAAVIGGYMAMNIGANDVSNNVGPAVGSKAISMGGAILIAAICEMLGAI
             :   :        * :*.:* ... : :  :    * ::* . :  *.

Y64o_       LAGGEVTQTIKSGVIEPIQF-----------VDTPDILALGMLSTLFAS
Pho4_       LLGHQVTDTMRKGVIDFSEFGKHENGTVLMSENDLGHTIMLGQIAILTGC
PHO89_      LAGARVSGTIKNNIIDSSIF-----------TNDPAVLMLTMTSALIGS
PitB_       LGGLSVAYAIVHMLPTDLLLN----------MGSTHGLAMVFSMLLAA
PitA_       LGGLSVAYAIVHMLPTDLLLN----------MGSSHGLAMVFSMLLAA
HP1491_     IAGGEVVSTIKGRIVSPEFIN----------DAHIFINVMLASLLSG
            : *  *  ::   :     :                       : * .

Y64o_       GAWLFIATKMGWPVSGTHTIIGAIIGFACITIGPS------SVDWSKIGS
Pho4_       GVWMLLATAFKLPVSTTHSIVGATIGFALVAQGSR------VIVWEKIYR
PHO89_      SCWLTFATAIGMPVSTTHSIVGGTIGAGIAAGGANGV----VWGWSGVSQ
PitB_       IIWNLGTWFFGLPASSSHTLIGAIIGIGLTNALLTGSSVMDALNLREVTK
PitA_       IIWNLGTWYFGLPASSSHTLIGAIIGIGLTNALMTGTSVVDALNIPKVLS
HP1491_     ALWLHVATLIGAPVSTSHSVVGGIMGAGMAAAGMV------AVNWHFLSG
              *   :  :  *.* :*:::*. :* .                  :

Y64o_       IVGSWFVTPVIAGILAYAIFASTQKLIFDT-----------EQPLKNAQK
Pho4_       IFFSWIISPLLSGIVSVFIYLSLDHLVLRR-----------EQPLHSGIR
PHO89_      IIASWFIAPILAGAIAAIVFSISRFSVLEVKSL--------ERSIKNALL
PitB_       IFSSLIVSPIVGLVIAGGLIFLLRRYWSGTKKRDRIHRIPEDRKKKKGKR
PitA_       IFGSLIVSPIVGLVFAGGLIFLLRRYWSGTKKRARIHLTPAEREKKDGKK
HP1491_     IVASWVISPLMGALIAMFFLMLIKKTIAYK-----------EDKKSAALK
            *. * .::*::.  .:  .                       :     .

Y64o_       YGP--YYMGITVFVLCIVTMKKGLKHVGLNLSNSETLIIS----LAISLI
```

```
Pho4_      VLPALYFICFAFNVFAIVYKGPSFLYFDR-LSLTQCLIISGVFGLAVALI
PHO89_     LVGVLVFATFSILTMLIVWKGSPNLHLDD-LSETETAVSIVLTGAIASIV
PitB_      KPPFWTRIALIVSAAGVAFSHGANDGQKGIGLVMLVLVGIAPAGFVVNMN
PitA_      KPPFWTRIALILSAIGVAFSHGANDGQKGIGLVMLVLIGVAPAGFVVNMN
HP1491_    VVPYLVALMSLTFSWYLIVKVLKRLYALN----FEIQLACG---CILALL

Y64o_      ------------AVEKVFSILMLLTACAMAFAHGSNDVANAIGPLSAVVS
Pho4_      ASFFRSCKPEDPQASRLFSLLQVMTACFGGFAHGGNDVSNAIAPLVSLYL
PHO89_     KGMYERSKFYDNRVEYIYSVLQAITAATMSFAHGANDVANATGPLSAVYV
PitB_      -----NMESYEPLSVSQRSQLRRIMLCISDTSAKLAKLPGVSKEDQNLLK
PitA_      -----DVESYDKLSLDQRSQMRRIMLCVSDTIDKVVKMPGVSADDQRLLK
HP1491_    -------------INELFNVPLIFAAALLSFAHGANDVANAIGPLAAISQ

Y64o_      IVNEG--GKIVSGGALTWWILPLGALGIAVGLITMGQKVMATVGSGITDL
Pho4_      IANDG--MKSAD-METPWYLLLYGSFGMCLGLWVLGHRVIYTVGENLTKI
PHO89_     IWKTN--TIGAK-SEVPVWVLAYGGVALVIGCWTYGYNIIKNLGNKMILQ
PitB_      KLRSD--MLSTI-EYAPVWIIMAVALALGIGTMIGWRRVAMTIGEKIGKR
PitA_      KLKSD--MLSTI-EYAPVWIIMAVALALGIGTMIGWRRVATTIGEKIGKK
HP1491_    TLEDANSPIGNTLSSVPLWIMVVGAAGIALGLSLYGPKLIKTVGSEITEL

Y64o_      --TPSRGFAAQFATAMTVVVASGTGLPISTTQTLVGAILGIGFA-R----
Pho4_      --TPASGFAVEFGAAVTVLIASKLGLPISSTQCKVGSVVAVGLV-Q----
PHO89_     --SPSRGFSIELAVAITTVMATQLGIPTSTTQIAVGGIVAVGLCNK----
PitB_      GMTYAQGMAAQMTAAVSIGLASYIGMPVSTTHVLSSAVAGTMVV------
PitA_      GMTYAQGMSAQMTAAVSIGLASYTGMPVSTTHVLSSSVAGTMVV------
HP1491_    --DKMQAFCIALSAVITVLLASQLGLPVSSTHIVVGAVFGVGFLRERLRE

Y64o_      --------------------GIAALNLTVIRNIISSWIVTLPAGAFFA-
Pho4_      --------------------SRHQVHWGVFRNISLSWIVTLPVAGLLSG
PHO89_     --------------------DLKSVNWRMVAWCYSGWFLTLPIAGLIAG
PitB_      --------------------DGGGLQRKTVTSILMAWVFTLPAAIFLSG
PitA_      --------------------DGGGLQRKTVTSILMAWVFTLPAAVLLSG
HP1491_    KNTAIALELKKKEKKSLKKVYKEEVIKRSILKKIVTAWLVTVPVSALLG-

Y64o_      ---IIIFYVLR---------TIFN---
Pho4_      GT-MMILHLFSFGTDATPKDSILQLFF
PHO89_     IINGIILNAPRFG-------VEYQMT-
PitB_      GLYWIALQLI----------------
PitA_      GLYWLSLQFL----------------
HP1491_    ---ALLFVALG---------FIEKYF-
```

Figure. 4.1 Comparison of the deducted amino acid sequences of *H. pylori* HP1491 and PitA, PitB of *E. coli*, Y640 of *Haemophilus influenzae*, Pho4 of *Neurospora crassa*, Pho89 of *S. cerevisiae*. The alignment was constructed using the CLUSTAL W program in the Biology WorkBench Pakage.

Nucleotide Sequence:

>HP1491 (1599bp)

```
       1 atggaaatta aaaacatcaa agagtttgaa aaagcttcca aaaaactcca aaaagacact
      61 ttaaagatcg ctctcgctct tttgtttctc atcggtgccg ctttactcgc tctcatttt
     121 gggcaggcta attctaaggg attgttgctt atttttgcgg ccgtgattgg ggggtatatg
     181 gcgatgaata ttggcgcgaa tgatgtgtct aataatgtcg gccctgccgt aggctctaaa
     241 gccattagca tgggcggggc gattttgatc gctgcgattt gcgaaatgct tggagcgatc
     301 attgctgggg gggaagtggt ttctacgatt aagggccgta tcgtttcgcc tgaatttatt
     361 aatgatgcgc acattttcat taatgtcatg ttggctagct tactcagtgg ggcgttgtgg
     421 ttgcatgtag cgactttaat tggcgctccc gtttccactt cacactctgt ggtgggggg
     481 attatggggg ctggaatggc agcagctgga atggtagctg tcaattggca ttttttatcc
     541 ggtattgtgg ccagttgggt aatttcgcct ttaatggggg ctttgatagc catgtttttt
     601 ttaatgctca ttaaaaagac tatcgcttat aaagaagata aaaagagtgc ggctttaaag
     661 gtcgtgcctt atttggtagc gttgatgagc ttaacattca gctggtattt gattgttaag
     721 gttttaaaac gcctctatgc attgaatttt gaaatccagt tggcttgtgg ctgtatcctt
     781 gcgcttttaa tctttatcct ttttaaaaga tttgtgttaa agaaagcccc gcaattagaa
                                                  3'-ACACGGATCCCGCAATTAGAA
     841 aacagccatg aaagcattaa tgagcttttc aatgtccctt tgatttttgc cgctgcactt
         AACAGCCATG A-5'
     901 ttaagctttg cgcatggggc taatgatgtg gctaacgcta taggcccctt agccgcaatc
     961 agtcaaactt tagaagatgc aaatagccct atagggaata ctttaagctc tgtgccgttg
    1021 tggattatgg tagtaggggc ggctgggatt gctttaggct tgagtttgta tgggccaaag
    1081 ctcattaaaa cggtggggtc agaaatcaca gaattagaca agatgcaagc tttttgcatc
    1141 gcgctttcag cagtcatcac cgtgctttta gcctctcaat taggcttgcc cgtaagctct
    1201 acgcatattg tggtgggcgc ggtgttttgg gtgggctttt taagggagcg cttaagagag

                                    5'-ACACGGATCCTCTTAAGCGCTCCCTTAAAAAG-3'
    1261 caatcaagaa ggcgttttgc tagaatcaga gacaacattg tagccgcgca ctttggggaa
    1321 gatttagaag aaattgaagg ctttttagag cgctttgata aagccaattt gaaagaaaaa
    1381 tcgctcatgc tagagagctt aaaaaaaagc aagaacaccg ccatcgcttt ggaattgaaa
    1441 aagaaagaaa aaaagtcgct taaaaaagtg tataaagaag aagtgatcaa acgctccatt
    1501 ttaaaaaaga ttgttaccgc ttggttggta accgtgccgg tttctgcgct tttgggggcg
    1561 cttctgtttg tggctcttgg ttttatagaa aagtatttct ag
```

Protein Sequence:

>HP1491 (533aa)

```
       1 meiknikefe kaskklqkdt lkialallfl igaallalif gqanskglll ifaaviggym
      61 amnigandvs nnvgpavgsk aismggaili aaicemlgai iaggevvsti kgrivspefi
     121 ndahifinvm lasllsgalw lhvatligap vstshsvvgg imgagmaaag mvavnwhfls
     181 givaswvisp lmgaliamff lmlikktiay kedkksaalk vvpylvalms ltfswylivk
     241 vlkrlyalnf eiqlacgcil allifilfkr fvlkkapqle nshesinelf nvplifaaal
     301 lsfahgandv anaigplaai sqtledansp igntlssvpl wimvvgaagi alglslygpk
     361 liktvgseit eldkmqafci alsavitvll asqlglpvss thivvgavfg vgflrerlre
     421 qsrrrfarir dnivaahfge dleeiegfle rfdkanlkek slmleslkks kntaialelk
     481 kkekkslkkv ykeevikrsi lkkivtawlv tvpvsallga llfvalgfie kyf
```

Figure 4.2. Nucleotide sequence and the deduced amino acid sequence of the HP1491 gene. The primer for PCR was indicated by arrow. The sequence data reported in this paper have been deposited with the GenBank/EMBL databank under accession ID AADO8533.1.

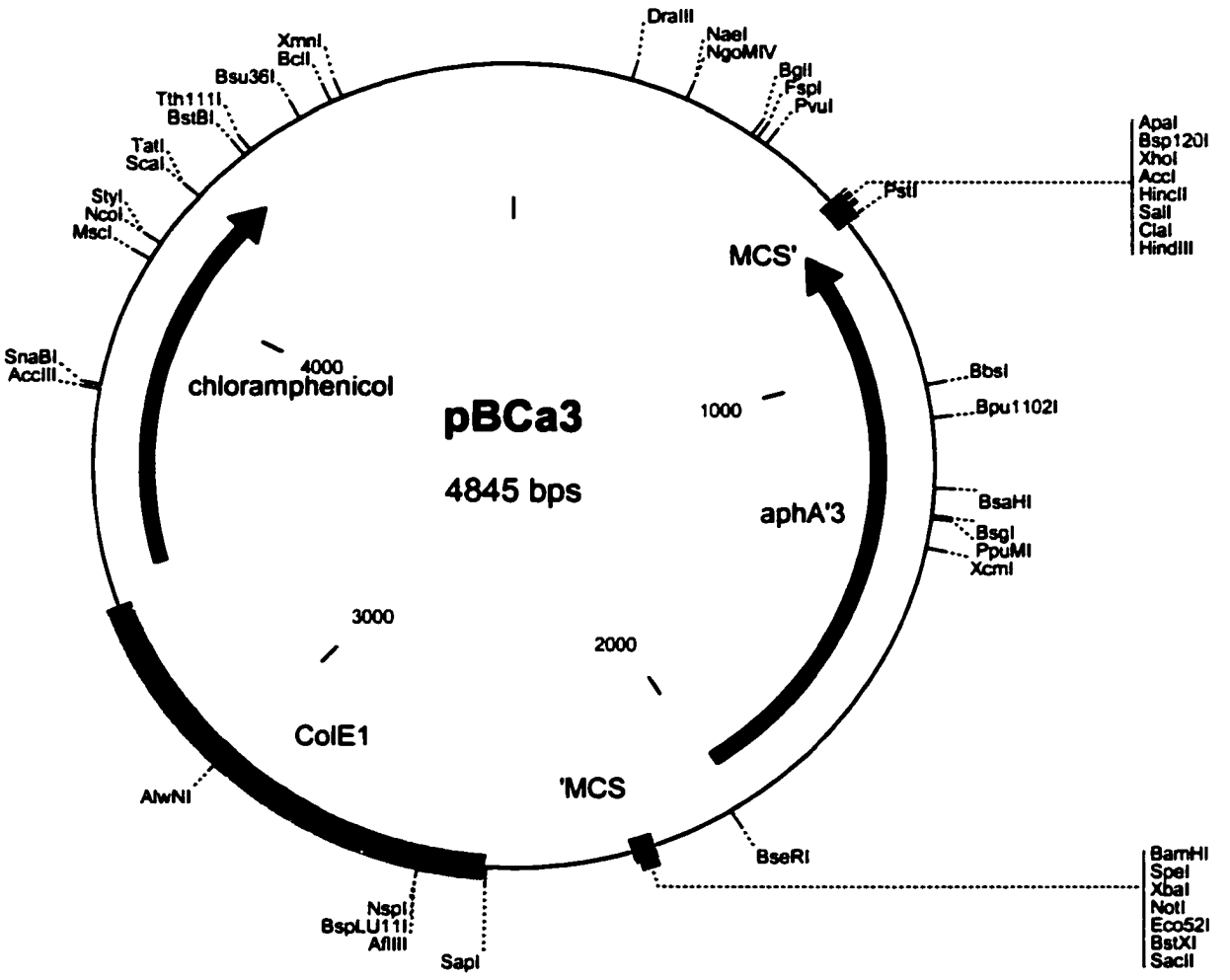


Figure. 4.3. Schematic representation of pBCα3. pBCα3 plasmid contains ColE1, the origin of replication which is functional in *E. coli* but not in *H. pylori*; pBCa3 also has the aphA-3 kanamycin resistance cassette which contains a promoter and a Shine-Dalgarno site, which are fully functional in *H. pylori* and *E. coli*.

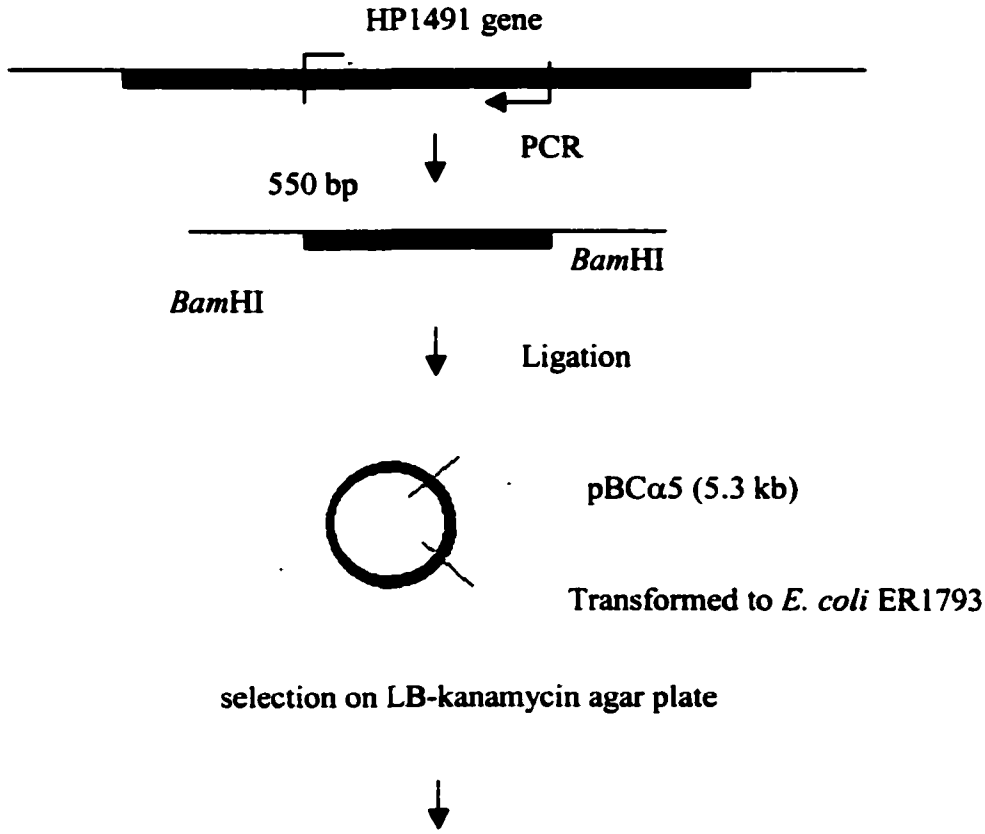
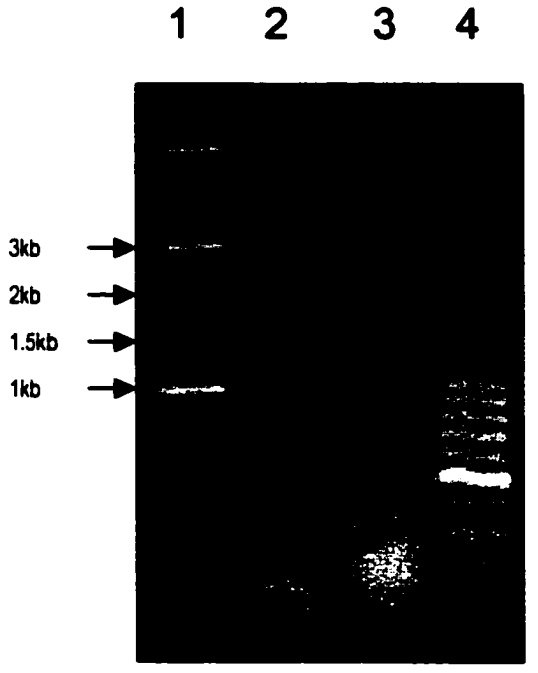


Figure 4.4. Cloning of HP1491 gene to suicide vector pBCα3. A fragment of HP1491 gene of 550 bp gene was amplified by PCR with *Bam*HI restriction sites on both the amino terminal and carboxyl terminal primer. PCR product was dephosphorylated and ligated to pBCα3 with *Bam*HI digestion. The recombinant plasmid was transformed to ER1793 by electroporation, and selected on LB-kanamycin agar plate.

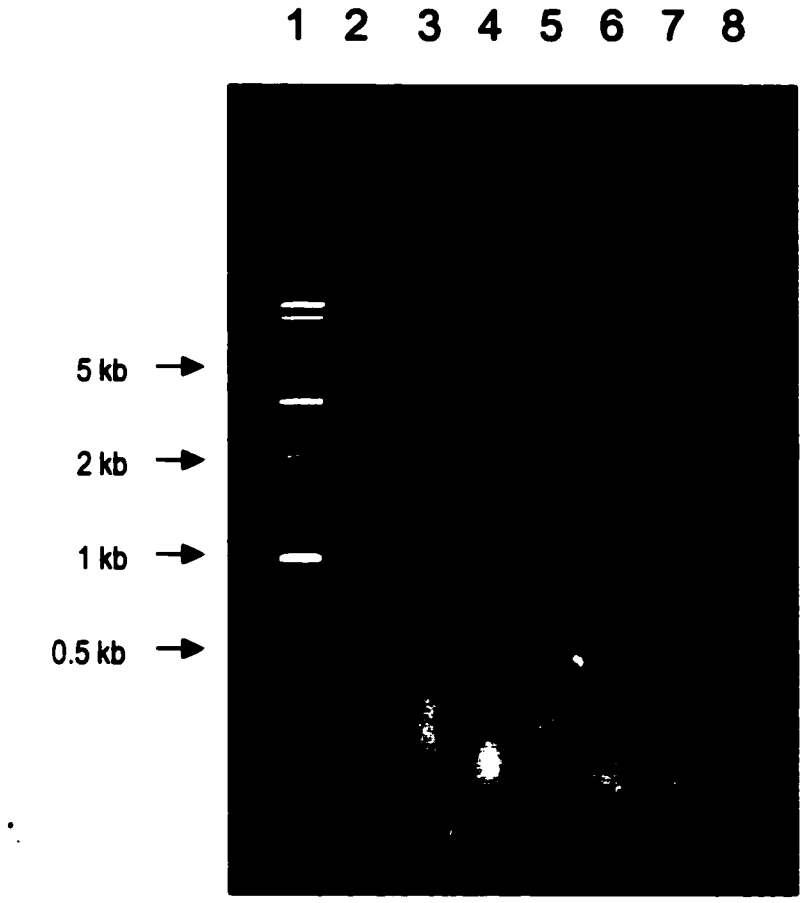


Expected restriction fragments

Enzyme Fragment	*Hind*III/*Bam*HI	*Hind*III/*Not*I
Without insert	1.5 kb 3.3 kb	1.5 kb 3.3 kb
With insert (recombinant)	2 kb 3.3 kb	2 Kb 3.3 Kb

Lane 1: DNA 1 kb marker
Lane 2: RE of pBCα5 by *Hind*III/*Not*I (2 kb, 3.3kb, with insert)
Lane 3: RE of pBCα5 by *Hind*III/*Not*I (1.5 kb, 3.3kb, without insert)
Lane 4: DNA 100 bp marker

Figure 4.5.1 Restriction analysis of the recombinant vector pBCα5.



Expected restriction fragments

Lane 1: DNA 1 kb marker
Lane 2: PCR product 550 bp
Lane 3: RE of pBCα5 by *Bam*HI (5.3 kb with insert)
Lane 4: RE of pBCα5 by *Hind*III/*Not*I (2 kb, 3.3kb)
Lane 5: RE of pBCα5 by *Bam*HI (5.3 kb with insert)
Lane 6: RE of pBCα5 by *Hind*III/*Not*I (2 kb, 3.3kb)
Lane 7: RE of pBCα5 by *Hind*III/*Not*I (2 kb, 3.3kb)
Lane 8: RE of pBCα5 by *Bam*HI (5.3 kb with insert)

Figure 4.5.2 Restriction analysis of the recombinant vector pBCα5.

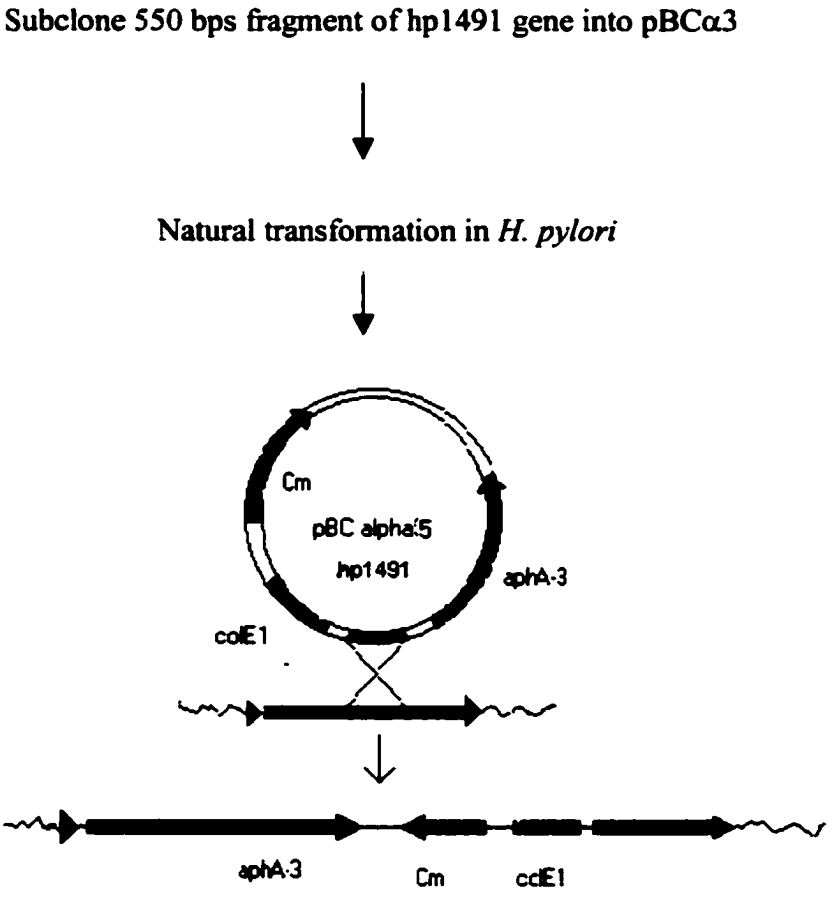


Figure 4.6. Schematic representation of the integration of pBCα5 in the chromosome. HP1491 DNA fragment (pink color) cloned into the plasmid mediated the integration of the plasmid into *H. pylori* chromosome via homologous recombination. Integration of pBCα5 results in a duplication of fragment HP1491 gene at both sites of insertion and a subsequent disruption of the gene in which HP1491 gene fragment is present.

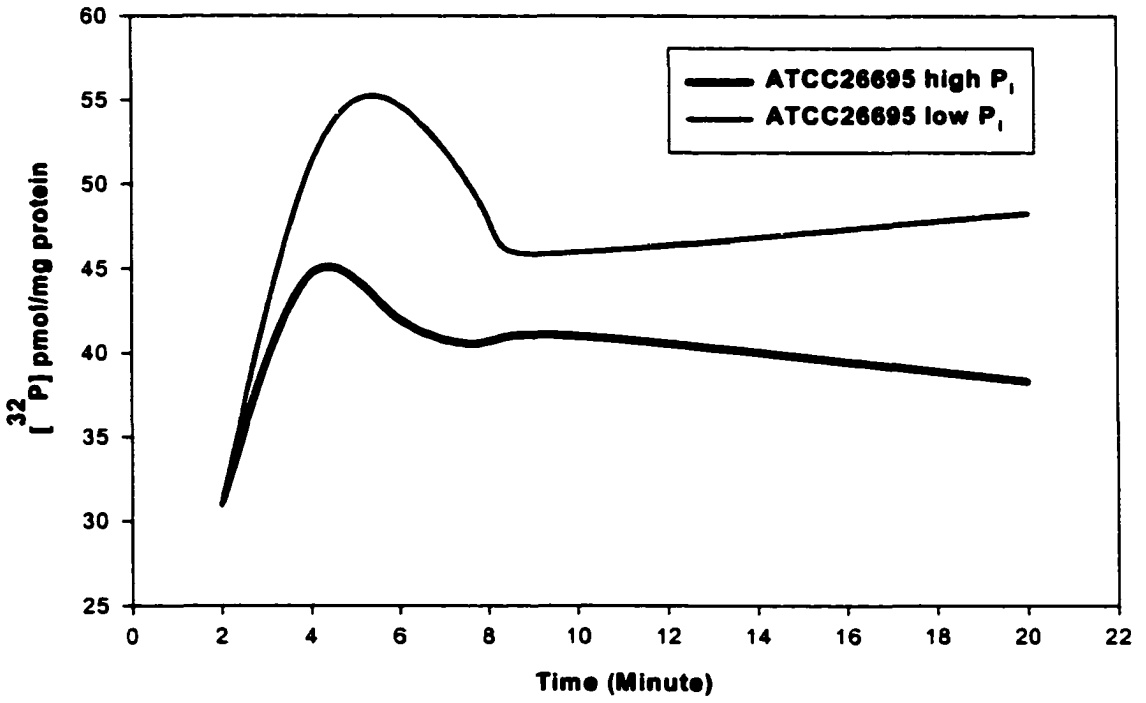


Figure. 4.7. Phosphate uptake by wild-type (ATCC 26695) cells of *H. pylori*. Cells grown in low-P_i medium (—) and cells grown in high-P_i medium (—) were assayed for P_i transport activity at pH 7.0 as described in Materials and Methods.

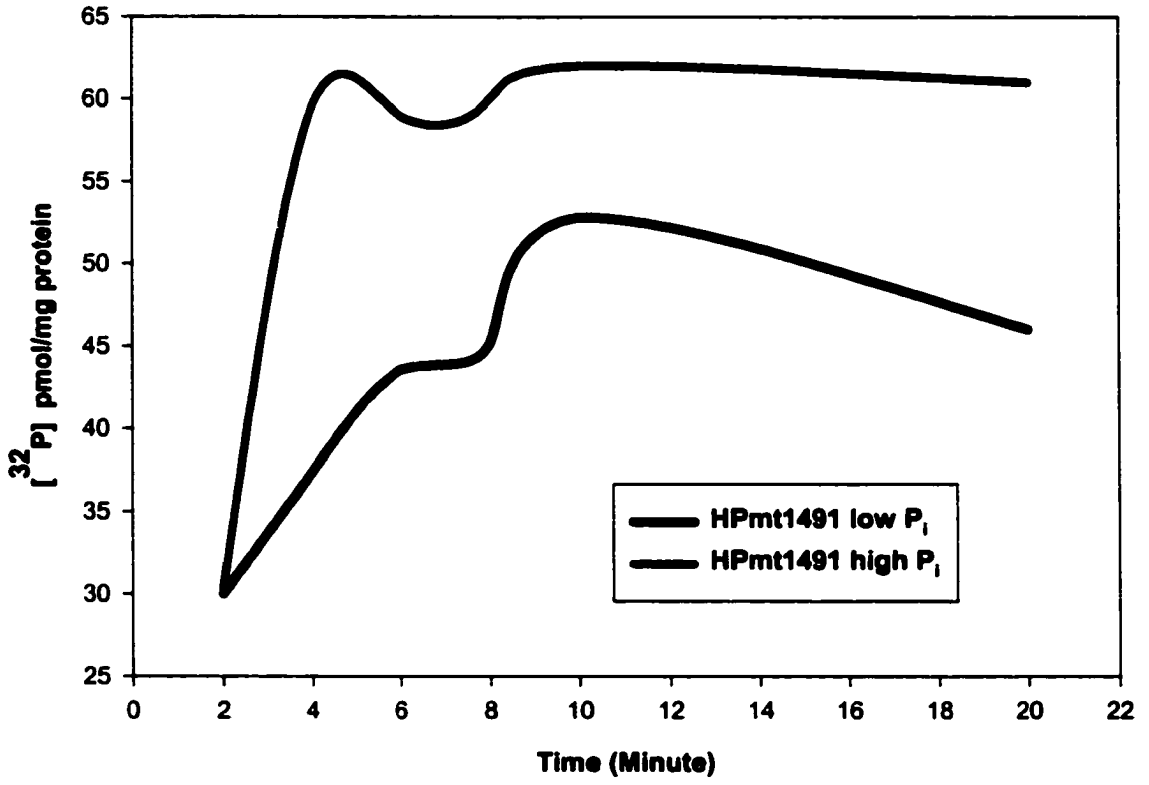


Figure. 4.8. Phosphate uptake by mutant (HPmt1491) cells of *H. pylori*. Cells grown in low-P_i medium (—) and cells grown in high- P_i medium (—) were assayed for P_i transport activity at pH 7.0 as described in Materials and Methods.

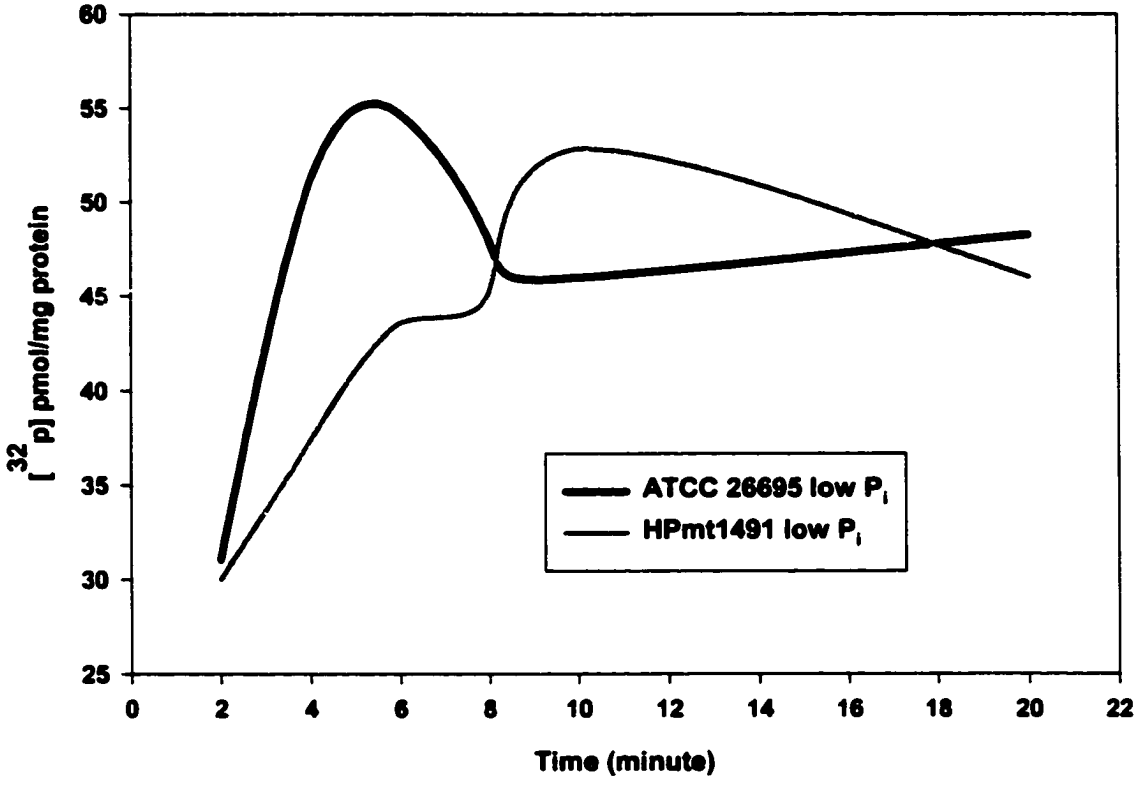


Figure. 4.9. Phosphate uptake by cells of *H. pylori* at low phosphate medium. Cells grown in ATCC 26695 (—) and cells grown in HPmt1491(—) were assayed for P_i transport activity at pH 7.0 as described in Materials and Methods.

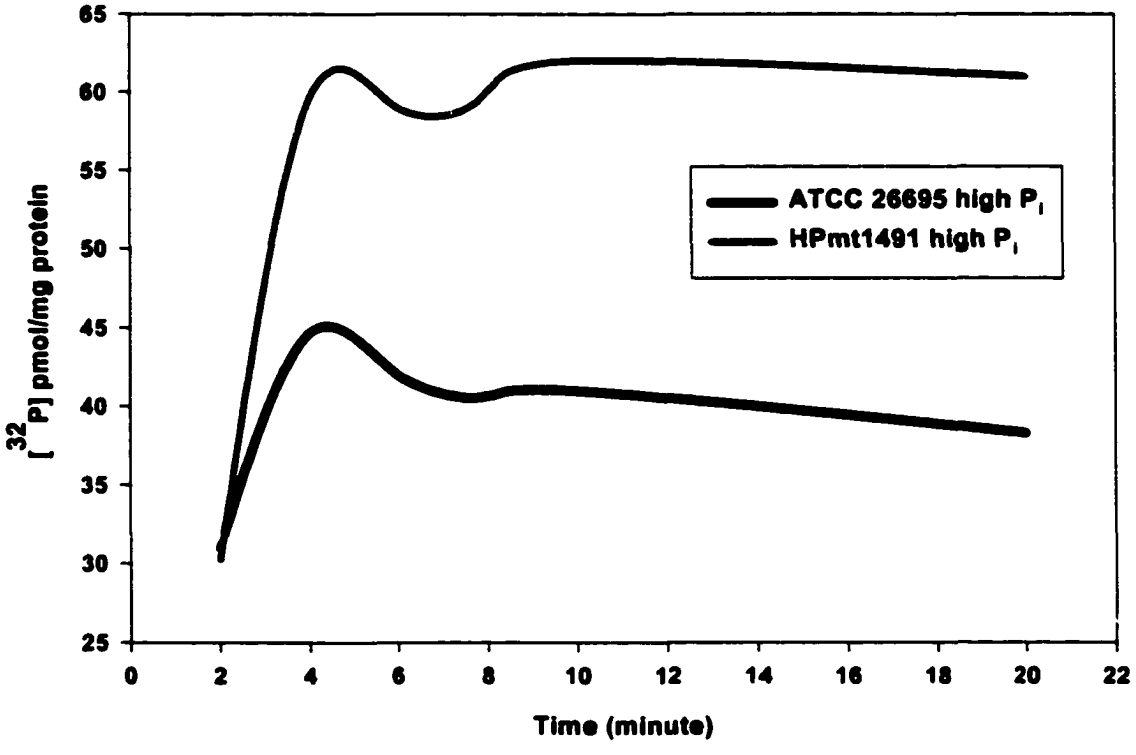


Figure. 4.10. Phosphate uptake by cells of *H. pylori* in high phosphate medium. Cells grown in ATCC 26695 (—) and cells grown in HPmt1491(—) were assayed for P_i transport activity at pH 7.0 as described in Materials and Methods.

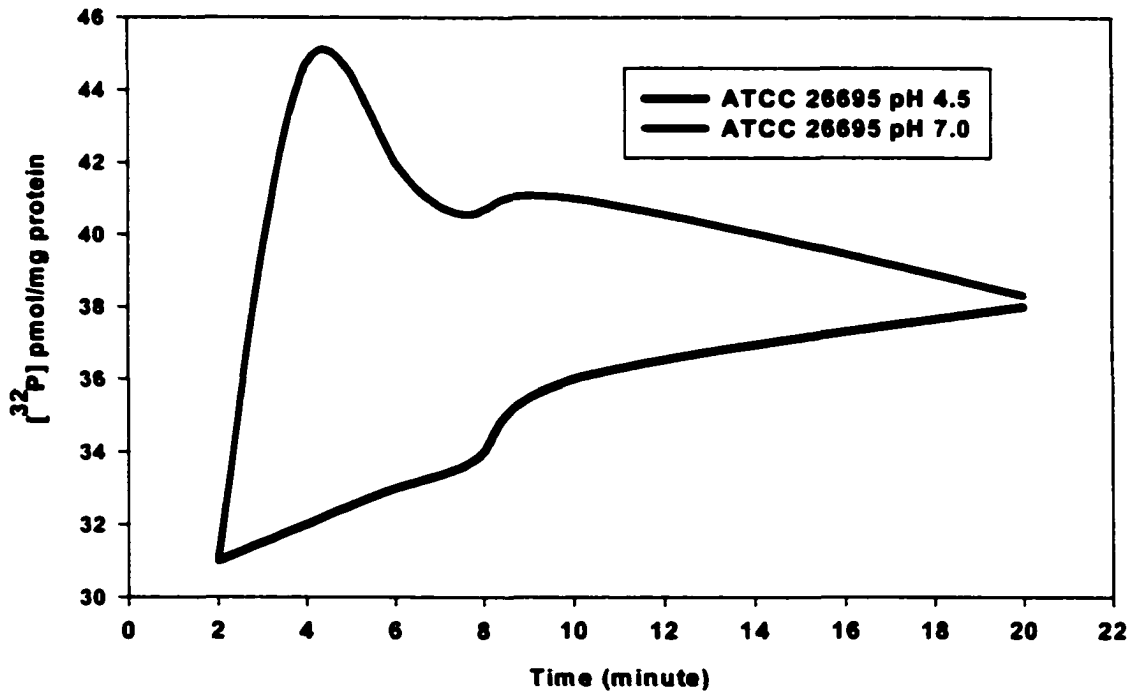


Figure. 4.11. pH effect on phosphate uptake of ATCC 26695 at high-phosphate medium. The external pH of *H. pylori* cells grown in high-P_i medium was varied by resuspending in Tris-succiniate buffer with the desired pH value. Cells grown at pH 4.5 (—) and cells grown at pH 7.0 (—) were assayed for P_i transport activity as described in Materials and Methods.

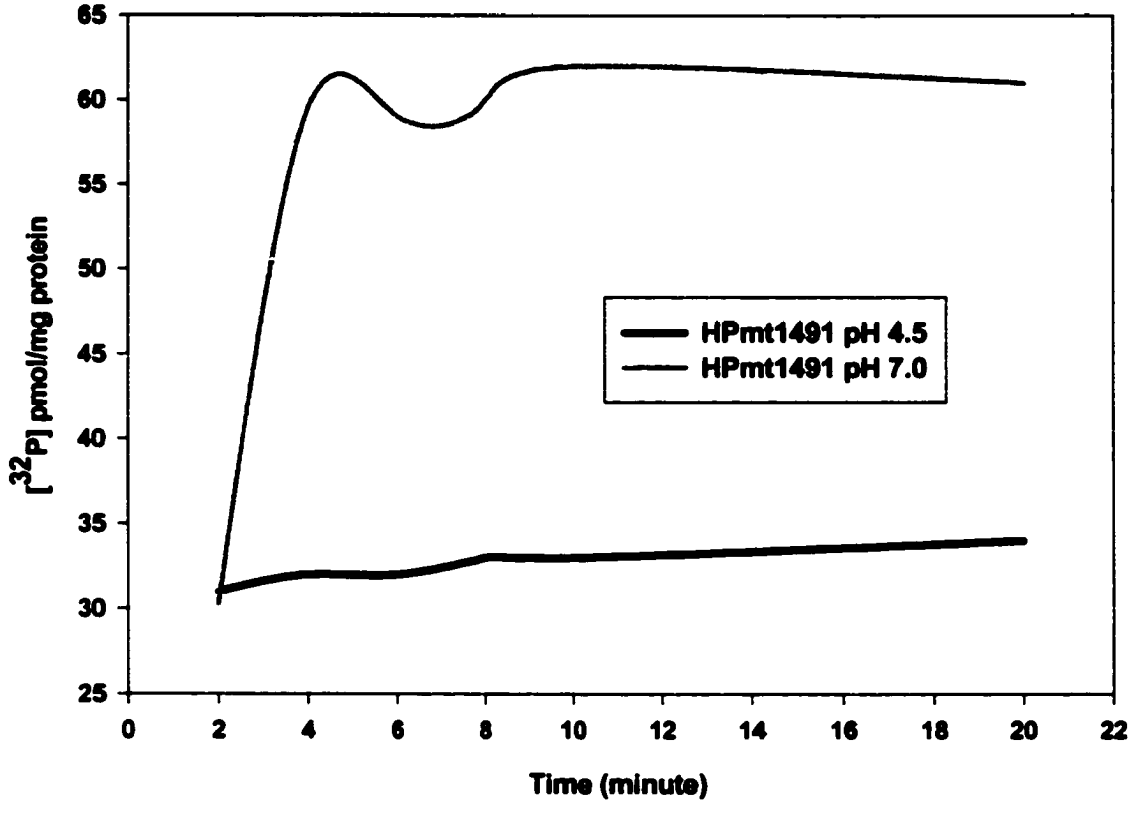


Figure. 4.12. pH effect on phosphate uptake of HPmt1491 at high-phosphate medium. The external pH of *H. pylori* cells grown in high-P_i medium was varied by resuspending in Tris-succinate buffer with the desired pH value. Cells grown at pH 4.5 (—) and cells grown at pH 7.0 (—) were assayed for P_i transport activity as described in Materials and Methods.

Results

A computer search using the BLAST algorithm revealed several protein sequences that produced high-scoring segment pairs with the HP1491. Among these, several protein sequences were related to proposed phosphate transport proteins, namely Y640 (phosphate permease) of *Haemophilus influenzae*, Pho4 (Na^+/P_i cotransporter) of *Neurospora crassa*, Pho89 (Na^+/P_i transporter) of *S. cerevisiae*, and PitA and PitB of *E. coli*. When HP1491 was compared with Pho89, another proposed high-affinity plasma membrane Na^+/P_i transporter, it revealed a striking overall identity to the amino acid sequence of 38% and a similarly of 59% (figure. 4.1). It therefore seemed likely that the HP1491 is a phosphate transporter in *H. pylori*.

Figure 4.2 showed the nucleotide and deduced protein sequences of HP1491. Bold, underlined letters indicates the location of the PCR primers. The actual primer sequences, including the *Bam*HI sites were interjected into the sequence (particle rows). The pBCα3 integration, suicide plasmid map was shown in figure 4.3. The unique *Bam*HI insertion site was located in the lower multiple cloning sites.

The PCR product of HP1491 was digested with *Bam*HI and cloned into ER1793 (figure 4.4), also agarose gel (figure 4.5.1) showed the restriction enzyme *Hind*III/*Not*I digestion of pBCα3 with insert (lane 2, 2 kb and 3.3 kb) and without insert (lane 3, 1.5 kb and 3.3 kb); another agarose gel (figure 4.5.2) showed the digested pBCα5 (lane 3, 5.3 kb), the PCR fragment (lane 2, 550bp) to be inserted into the multiple coning site (lane 4, 2 kb, 3.3 kb), and the product of the ligation and transformation into *E. coli* (figure 4.4). PCR amplification product of HP1491 gene after insertion of pBCα5 w/insert was shown

in figure 4.5.2. Compared to HP1491 PCR product (lane 2, 550 bp), the insertion increased the size of the PCR product to 2kb (lane 4).

Characterization of the HP1491 mutant strain was accomplished by assaying phosphate uptake for the first 20 minutes after phosphate starvation as compared to ATCC 26695 wild type strain. Figure 4.7 revealed several important characteristics of phosphate uptake in the wild type organism. First, there was a very rapid initial rate followed by a burst of phosphate uptake lasting 6-10 minutes. Second, the initial rate and the peak of the burst were much grater in cells grown on low phosphate media, as compared to cells grown on high phosphate media. And third, in both media types, the burst ends after 6-8 minutes and the rate resumed, regulated phosphate uptake, as if in the burst the cells had overcompensated for the starvation period and the regulatory mechanisms were then necessary to obtain the normal rate of uptake.

When the phosphate permease HP1491 was mutated, the pattern of phosphate uptake in starved cells changed (see figure 4.8). Mutant cells in low phosphate media had a reduced initial rate of uptake and a delayed burst, however, the magnitude of the burst and the regulated uptake region were not very different from wild type (figure 4.9). On the other hand, mutant cells grown on high phosphate media exhibited initial rates and timing of the burst that were similar to wild type (figure 4.10), but the magnitude of the peak activity and the regulated region were 25-35 % higher. Also, the burst was not well defined.

To define the activity of the phosphate permease on low and high phosphate media, the activities of the various strains were compared and the differences in curves summarized (figure 4.7 – figure 4.10). On low phosphate media, the permease appeared

to play a role in maintaining the initial rate, and possibly in regaining control after the burst. In high phosphate media, the control over the magnitude of the reaction was lost and so was regulation after the burst.

Comparison of phosphate uptake activity in wild type and mutant strains from neutral and acidic (pH 4.5) media were shown in figure 4.11 and figure 4.12, under acidic conditions, the phosphate permease appeared to be responsible for all of the phosphate activity that remains, including the hint of a burst that disappeared in pH 4.5.

These experiments have allowed us to partially define the possible activity of the phosphate permease and to know that the permease is not the only system for obtaining phosphate in wild type *H. pylori* cells. The permease appears to be responsible for maintaining the low activity observed during low pH and it seems to have some role in the initial rate of activity, and possible in recovery of rates after the burst; high phosphate cells do not maintain the magnitude of wild type nor do they seek a lower rate after the burst.

Discussion

The identification of the HP1491 gene as a phosphate permease was supported by a comparison of its deduced amino acid sequence with the sequence of a proposed phosphate transporter in the related organism *S. cerevisiae* and of other phosphate transporters of eukaryotic and prokaryotic origin. Phosphate uptake by *S. cerevisiae* has for long been known to be accomplished by high-affinity (Pho89, Na^+/Pi transporter) and low-affinity (Pho84, H^+/P_i transporter) systems. Based on the homology of HP1491 and Pho89, and other unidentified proteins that could be involved in phosphate transport

systems, such as HP1577 (ABC transporter, permease protein), HP1465 (ABC transporter, ATP-binding protein). It is presumed that *H. pylori* phosphate uptake systems may be similar to *S. cerevisiae*. Our preliminary results on the effect of the concentration of phosphate on phosphate uptake rate (figure 4.7, 4.8) corroborate the sequence data and it seems likely that at least two separate phosphate transport systems, with distinct pH optima (figure 4.11, 4.12) and phosphate affinities exist in *H. pylori*.

In the wild type organism (ATCC 26695), phosphate uptake under conditions of high and low phosphate shows that there is tight control as seen in other organisms. In *E. coli* and *Saccharomyces*, the control has been shown to come from the fact that two distinct phosphate uptake systems exist; one is active at high phosphate concentrations, the other at low phosphate concentrations, exclusively. From the data obtained from the wild type strain, this could be true of *H. pylori* also. But mutating HP1491 gave results that are not consistent with this hypothesis. If the product of HP1491 is central to one of the two systems, then either on low or high phosphate there should be a major reduction in uptake. Instead, on low phosphate the net uptake is in the normal range and on high phosphate the uptake is extremely high compared to any uptake by the wild type. It appears that mutating HP1491 has removed a control that regulates the total phosphate uptake as if something about HP1491 controls feedback of all phosphate uptake systems.

On the other hand, the data from growth of HPmt1491 in acid shows that HP1491 is responsible for the increase in phosphate uptake under acid conditions. While its activity is slower under acid conditions, it is more resistant to acid than the other system(s).

Chapter Five

DISCUSSION

The discovery of a bacterium living in the human stomach was unexpected and presented new and interesting questions for both clinical and basic microbiology. Prior to this discovery ulcer patients suffered for years, sometimes their entire lives, because it was not expected that a bacterium could survive, and even thrive, under such harsh conditions. It is interesting that this discovery took place at a time when the study of environmental microbiology was undergoing rapid changes and we were on the verge of discovering how adaptive microbial life can be with the study of the extremophiles; evolution has allowed the adaptation of life to all environments, no matter how harsh.

Clinicians were faced with how to kill an organism that can virtually hide from the lumen of the stomach, seeking refuge under the mucus, at the cell surface. And yet in most cases, the organism is not in the tissues; it is actually outside the body from the standpoint of circulating antibiotics in high enough concentrations to kill. However, when antibiotic treatment was successful, they were able to cure recurrent ulcers that had caused patients to suffer for decades.

The epidemiology of the fastidious *Helicobacter pylori* has presented a challenge as well. The organism does not survive well outside of the body; in fact, it is very difficult to cultivate on the richest artificial media. Since humans do not usually share stomach contents, how is this infection transmitted? The information available so far is that *Helicobacter* colonization/infection is commonly found in members of the same

family, so either, it is transmitted by intimate contact or there is a common source that contacts all family members, such as a food source, though none have been identified as yet.

On the microbiology front, *Helicobacter pylori* is unique in that it lives in a part of the body that is so harsh that no other known organisms can live. Pathogenic mechanisms include its survival tactics such as: its ability to withstand low pH using urease to create a "cloud" of ammonia around itself, and its flagella and adhesins that allow it to swim and bind to mucins to enter its safe niche, the mucous lining of the stomach.

Helicobacter pylori is especially interesting since it is not an acidophile, so unlike the adaptations found in nature, this organism has not re-developed its entire physiology to survive but instead it has developed specialized mechanisms for tolerating low pH when necessary. It is similar in this respect to all of the organisms of the stomach. They must pass through the stomach to reside in the intestine and they too have developed specialized mechanisms for tolerating a brief period in the gastric juice. The study of *H. pylori* and its survival strategies stands to enlighten us on these special adaptations of the acid tolerant organisms.

In this study, we observed a major reduction in, if not a complete shut down of, phosphate uptake in the presence of acid. How can the cell survive without continual phosphate uptake? There are several feasible explanations. First, the cell may go into a dormancy-like state, shutting down most cellular functions while in acid. Second, in all likelihood, there is another source of phosphate in the cell. The cell continues with normal function as evidenced by the data on protein synthesis, although it slows down as

the pH is lowered. It is possible that intracellular stores of phosphate (polyphosphates) could serve as a source of phosphate for the cell during phosphate starvation. However, the cell must have an external source of phosphate to survive for extended periods such as *H. pylori* living in the stomach under acid conditions. By mutating HP1491, the putative phosphate permease, we observed that the pattern of phosphate uptake was modified under both neutral and acid conditions. The fact that the cells are still viable, but change their pattern of uptake of phosphate, indicates that HP1491 is, in fact, a phosphate permease but it is not the only phosphate uptake mechanism. The system or systems of phosphate uptake that remain in the mutant strain are still active at low pH, however, the rate and the initial burst of uptake change dependent upon which system is active.

Is HP1491 activity, in fact, controlled by acidity and why? It seems that the cell would need phosphate in the cytoplasm for use in metabolism, but even more for its buffering capacity during growth in acid. Is the permease a proton symporter and therefore entry of every molecule of phosphate includes entry of a proton? In this case it seems logical that it would have to shut down under acid stress. Are other proteins involved in the function of HP1491?

To further understand phosphate uptake and metabolism, it will be necessary to collect and characterize other mutants of the phosphate uptake systems and determine their role in the toleration response to acid. This understanding will surely afford a better understanding of all acid tolerant organisms and their strategies and adaptations for survival.

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