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Characterization of Beta-lactam Resistance in the Gastric Pathogen *Helicobacter pylori*

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ABSTRACT OF THE DISSERTATION

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by

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Helicobacter pylori is a major cause of peptic ulcer disease and potentially stomach cancer. Treatment of patients with gastritis is warranted and normally done with a combination of antibiotics (such as amoxicillin, clarithromycin or others) and a proton pump inhibitor. Resistance to most of these antibiotics in *H. pylori* has been seen worldwide and is constantly increasing.

The purpose of this thesis was to more systematically characterize the mechanisms of amoxicillin resistance in *H. pylori*. We used both a clinical isolate strain B258, the first US amoxicillin resistant strain to be examined in this way, and in vitro selected amoxicillin resistant strains. In strain B258 we determined that all of the amoxicillin resistance was due to the presence of 2 amino acid substitutions in Penicillin Binding Protein 1(PBP1). To explain how these amino acids could increase amoxicillin resistance, we did homology modeling of PBP1 to map the location of these amino acids and their relation to the putative amoxicillin binding cleft.

To better appreciate the role of single amino acid substitutions in amoxicillin resistance, we made site-directed mutants of the most commonly found amino acids in

PBP1 from amoxicillin-resistant strains. and transformed a sensitive strain with each to monitor its effect on amoxicillin resistance. We also used homology modeling and affinity binding studies using biotinylated amoxicillin to examine the role of these PBP1 mutations in amoxicillin resistance.

Finally, we examined a series of 6 in vitro selected amoxicillin resistant strains each with slightly increased MICs to amoxicillin, in order to track both the evolution of resistance as well as identify other possible novel resistance mechanisms. Whole genome sequencing of the most resistant isolate when compared to the parental sensitive strain, identified 12 genes as likely candidates affecting amoxicillin resistance. Changes in PBP1, PBP 2 and various outer membrane proteins were identified and their role in amoxicillin resistance is being systematically examined in each of the 6 isolates. Although studies are on-going, results to date have shown that in addition to PBP1, changes in outer membrane permeability are required to get high level amoxicillin resistance.

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Chapter 1

Background and Literature Review

Helicobacter pylori: History and prevalence

Helicobacter pylori (*H. pylori*) is a spiral-shaped, flagellated, Gram negative bacterium which colonizes the mucus layer of the gastric epithelium and is associated with chronic type B gastritis. Most infected individuals are asymptomatic (Malfertheiner et al., 2007), however infection is associated with the development of numerous gastric pathologies, including gastric ulcers, gastric adenocarcinoma and B-cell mucosa-associated lymphoid tissue (MALT) lymphoma (Dunn et al., 1997). It is the most common cause of Peptic Ulcer Disease (PUD) (Rosenstock et al., 2003) and is responsible for 5.5% cases of human cancer each year (Parkin, 2006). *H. pylori* has been recognized as a definitive bacterial carcinogen for humans by the International Agency for Research on Cancer, a World Health Organization subunit (Int. Agency Res. 1994). There is also some evidence to suggest that *H. pylori* may infect other organs including the liver (Dunn et al., 1997). It has also been associated with Alzheimer's disease (Malaguarnera et al., 2004).

Evidence suggests that *H. pylori* has infected humans for tens of thousands of years, and it has been hypothesized that *H. pylori* colonization may have been beneficial to human carriers hence providing a selective advantage (Falush et al., 2003; Ghose et al., 2002). Today, however *H. pylori* infections are responsible for a high number of morbidity and mortality rates. The discovery of *H. pylori* in 1982 by Warren and Marshall (Marshall et al., 1984) has had a tremendous effect on the treatment of stomach diseases by changing several gastric diseases from chronic relapsing diseases into a curable infectious disease. By self-ingestion experiments, they showed that it indeed

causes gastroduodenal disorders thereby fulfilling Koch's postulates (Warren et al., 1983). Previously, gastro-intestinal diseases such as gastritis had been attributed to stress and hyperacidity of the stomach.

Currently, the rate of *H. pylori* infection in middle-aged adults is 20-50% in industrialized countries and 80% or more in many developing countries (Frenck et al., 2003; Rothenbaucher et al., 2003). The prevalence in either is higher in people of low socioeconomic status (Frenck et al., 2003). Although there is no consensus yet on the mode of transmission, it has been speculated that it takes a fecal-oral or an oral-oral route (Mitchell et al., 1989). It has also been shown that *H. pylori* can be cultured from feces, polluted waters, gastric juices and even become airborne during efflux of gastric juices (Parsonnet et al., 1999; Thomas et al., 1992; Young et al., 2000).

Pathogenesis

H. pylori possesses a number of unusual characteristics, making its mode of colonization and pathogenesis interesting to study. In many ways it behaves like a commensal, as infected individuals are often asymptomatic (Megraud et al., 2007). The incidence of disease after colonization is thought to depend on the virulence of the infecting strain, host genetics and environmental factors (Van Amesterdam et al., 2006). Motility of *H. pylori* is by means of polar sheathed flagella by the expression of FlaA and FlaB (Josenhans et al., 1995), and is essential for establishing a persistent infection (Eaton et al., 1992). *H. pylori* is well adapted to the highly acidic conditions found in the gastric lumen by means of a potent urease enzyme which hydrolyzes urea into ammonia

and CO_2 and creating a neutral micro environment allowing it to reach the mucus layer (Perez-Perez et al., 1992). The bacterium has also been shown to possess mucinase enzyme which allows it to disrupt the oligomeric structure of mucin probably further facilitating movement within the mucus layer (Windle et al., 2000). A pH gradient exists within the mucus layer, being very acidic toward the gastric lumen and almost neutral near the epithelial surface. *H. pylori* uses this gradient for spatial orientation and colonizes a zone located 0-25µm above the tissue surface (Schreiber et al., 2004). *H. pylori* lipopolysaccharide (LPS) is not highly immunogenic as LPS O antigen on some strains mimics the Lewis antigen found on human cells (Iver et al., 1998, Muotiala et al 1992). In addition, *H. pylori* Lipid A does not bind to TLR4 and the flagellin does not activate TLR5 (Akst, 2010).

There are a few different mechanisms by which *H. pylori* can bind to mucins and epithelial cells. The best described of these is BabA, which mediates binding of the organism to fucosylated Leb blood group antigen which is found on the surface of gastric epithelial cells and in gastric mucus (Clyne et al., 2007). Heterogeneity among *H. pylori* strains in the expression of the BabA protein may be a factor that contributes to different clinical outcomes among *H. pylori* infected individuals (Clyne et al., 2007). Persistent infection leads to an inflammatory response caused in part by bacterial toxins such as VacA and OipA. Presence of these toxins is associated with severity of disease and tissue damage to the gastric epithelium (Atherton et al., 1995; Yamaoka et al., 2006). Some *H. pylori* strains also have the cag pathogenicity island, a 40 kb fragment of chromosomal DNA that encodes 30 genes. It encodes a type IV secretion system that transports CagA

and other virulence factors. Long term exposure to cagA has been associated with the development of gastric cancer (Muotiala et al., 1992; Odenbreit et al., 2000).

Diagnosis and Treatment

Several invasive and non-invasive methods are available for the detection of *H. pylori* and the presence of pathogenic elements, each test having some advantage over the other. Some of the non-invasive tests in order of choice include IgG antibody detection in blood samples, stool antigen test and Urease Breath Test. Invasive tests usually consist of gastric biopsy through fiber optic endoscopy. Molecular techniques such as PCR can also be used on biopsy samples and stool samples to detect either the presence of *H. pylori* or specific pathogenic factors (Megraud et al., 2000).

Treatment using various antibiotics have shown effective eradication. Among the commonly used antibiotics are metronidazole, ciprofloxacin (flouroquinolone), clarithromycin (macrolide), and amoxicillin (β-lactam). Modes of action range from affecting DNA directly, blocking DNA replication, blocking protein synthesis and inhibition of bacterial cell wall synthesis, respectively. The current recommended treatment for *H. pylori* eradication includes two antibiotics and an antisecretory drug, a proton pump inhibitor such as omeprazole along with bismuth salts. The first choice of antibiotics include amoxicillin, clarithromycin and metronidazole. A 14 day treatment is considered more effective than a 7 day treatment. If the first line of treatment is ineffective then a bismuth-based quadruple therapy is recommended if previously not used (Malfertheiner et al., 2002). In patients with reflux esophagitis on long term acid

suppression, eradication of *H. pylori* infection decreases inflammation and reverses corpus gastritis (Kuipers et al. 2004). It has also been observed that in patients with low-grade lymphoma, eradication of *H. pylori* has resulted in tumor regression (European *Helicobacter Pylori* Study, 1997).

Antibiotic Resistance

As with other pathogens, resistance to antibiotics has already been observed in *H. pylori*. Antibiotic resistance to many of the antibiotics suggested for *H. pylori* treatment is well-known, with various levels of resistance noted (Megraud, 2005). The rate of resistance is higher in developing countries as compared to that in developed countries mainly due to the variation of prescribing patterns (Hu et al., 2007). The most important factor resulting in antibiotic resistance is poor patient adherence to the drug regimen (Dunn et al., 1997). In addition *H. pylori* is naturally competent (bacterial capability to take up naked DNA from the environment) with rates between 10^{-3} to 10^{-6} (Israel et al., 2000; Tsuda et al., 1993; Nadenskov-Sorensen et al., 1990), allowing the bacterium to take up resistance genes from other microorganisms. Moreover, the mutation rate of *H. pylori* is significantly higher than that of many other bacteria (Megraud et al., 2007). It has been determined that *H. pylori* lacks some homologs of excision repair and mismatch enzymes crucial in repairing point mutations during DNA replications (Wang et al., 1999). When these point mutations occur in genes encoding for the target of antibiotics, it lowers the affinity of the target for antibiotics. These factors allow for persistent *H. pylori* infections and are frequently the cause of treatment failure.

Metronidazole Resistance

Typically used in the treatment of anaerobic bacteria and certain protozoa, metronidazole is a synthetic antibacterial and antiprotozoal agent. Its mode of action involves creating nicks or breaks in the microorganism's DNA. It is suggested that in anaerobes and some microaerophilic bacteria, metronidazole is converted into an active form by the housekeeping protein pyruvate:ferredoxin oxidoreductase through a reducing reaction within the bacterial cell (Walsh, 2003). This enzyme is not present in humans, so it has been a drug of choice against *H. pylori*. Resistance to metronidazole in *H. pylori* results from mutations in *rdxA* and *frxA* genes encoding an oxygen insensitive NADPH nitroreductase and a NAD(P)H flavin oxido-reductase respectively (Goodwin et al., 1985). Inactivation by frame-shift, mis-sense, insertion or deletions in the *rdxA* gene is found in the majority of metronidazole resistant isolates (Goodwin et al., 1985). These mutations prevent the formation of active form of metronidazole, rendering these bacteria resistant to its effect.

Clarithromycin Resistance

The macrolide family of antibiotics, of which clarithromycin is a member, targets the 23S rRNA of the 50S ribosomal subunit and causes subsequent inhibition of protein synthesis (Walsh, 2003). Since the human ribosome is structured differently than its bacterial counterpart, this group of antibiotics has been proven effective in treating bacterial infections. Furthermore in the treatment of *H. pylori* infections, clarithromycin is the drug of choice because it causes the least amount of gastrointestinal tract irritation

and is stable in the acid pH of the stomach. However, in *H. pylori*, studies have found that only two mutated sites on the 23S rRNA molecule are necessary to confer resistance. Two adenine to guanine transitions at gene positions 2142 and 2143 impact bacterial sensitivity to clarithromycin (Megraud et al., 2005).

Tetracycline resistance

Tetracycline is often used as part of the three drug regimen used to treat *H. pylori* infections and is also a protein synthesis inhibitor. It works by binding the 16S rRNA of the 30S ribosomal subunit and prevent the docking of amino-acylated tRNA (Alekshun et al., 2007). Resistance to tetracycline can result from many mechanisms. Some of these mechanisms include active efflux, enzymatic inactivation of the drug and changes in 16S ribosomal RNA which result in inhibition of its binding to tetracycline. A point mutation in the primary binding site from 965-967 in 16S rRNA of *H. pylori* results in decreased binding of tetracycline to its target (Dailidiene et al., 2002). Other changes have also been implicated in tetracycline resistance in *H. pylori.* These mechanisms individually or in combination can confer resistance against tetracycline.

Gram negative cell wall structure; target of β-lactam antibiotics

Gram negative bacteria are distinguished from Gram positive bacteria because they do not retain crystal violet dye after alcohol decolorization in Gram stain, resulting in pink or red bacteria seen after the safranin counterstain (Gram, 1884). The cell wall of Gram negative bacteria consists of a number of layers, differing greatly in their chemical

composition. The inner most membrane is the cytoplasmic membrane followed by a thin peptidoglycan layer, different in composition and thickness from the Gram positive peptidoglycan layer. Outside of the peptidoglycan layer is the outer membrane which contains lipopolysaccharide, consisting of lipid A, core polysaccharide and O antigen. Porins also exist in the outer membrane which act like channels for small hydrophilic molecules. The space between the peptidoglycan layer and the outer membrane is the periplasmic space (Salton et al., 1996). The peptidoglycan layer consists of N-acetyl glucosamine and N-acetyl muramic acid with a pentapeptide side chain. These units are crosslinked, forming a peptide bond between D-alanine on one chain and diaminopimelic acid on the other chain in Gram negative bacteria. This cross linking function is performed by transpeptidase enzymes known as Penicillin Binding Proteins (PBPs). The transpeptidase domains of these enzymes utilize an active site serine to perform the catalytic reaction (Wilke et al., 2005). PBPs also have transglycosylase activity responsible for peptidoglycan polymerization and insertion into pre-existing cell wall especially during new cell growth. (Goffin et al., 1998; Born et al., 2006) . Some PBPs can perform both activities (bifunctional) while others are monofunctional.

β-Lactam mode of action

The primary goal of the studies presented in this thesis is to characterize the various resistance mechanisms of *H. pylori* to the β-lactam class of antibiotics, which includes several commonly used antibiotics such as penicillin, amoxicillin and ampicillin. β-lactam antibiotics are bacteriocidal and inhibit the synthesis of new peptidoglycan layer

in actively dividing cells. This class of drugs is characterized by a heteroatomic ring structure which consists of three carbon atoms and one nitrogen atom. It is a cyclic amide. β-lactams are efficient due to the stereochemical similarity of the β-lactam moiety with the D-alanine-D-alanine substrate (Wilke et al., 2005). It covalently binds to a putative catalytic serine in the transpeptidase domain of PBPs (Contreras-Martel et al., 2009) rendering it ineffective.

β-Lactam Resistance Mechanisms

β-lactam antibiotics represent >65% of the world antibiotic market (Poole, 2004). Although the prevalence rates of amoxicillin resistance of *H. pylori* in the US are generally low, rates as high as 33% have been seen in developing countries (Hu et al., 2007).

β-lactam antibiotics inhibit the growth of *H. pylori* by covalently binding to PBPs and then blocking cell wall synthesis. Resistance to these drugs in Gram-negative bacteria is mostly due to β-lactamase activity (Bush, 2001) that disrupts the β-lactam ring rendering the drug inactive. In *H. pylori*, however β-lactamase activity has not been established (Bina et al., 2000; DeLoney et al., 2000) and resistance seems to be mainly mediated by alterations to PBPs. Four PBPs have been identified using labeled antibiotics: PBP1, PBP2, PBP3 and PBP4 (DeLoney et al., 1999). These four PBPs are distinguishable in size when separated by SDS-PAGE, with sizes of 66, 63, 60 and 47 respectively (DeLoney et., 1999). Of these four the role of PBP1 has been extensively studied in conferring β-lactam resistance to *H. pylori* by our lab as well as others (Co et

al., 2006; Kwon et al., 2003; Gerrits et al., 2002 and 2006; Matteo et al., 2008; Okamoto et al., 2002; Paul et al., 2001 and Rimbara et al., 2007). PBP1 possesses two domains: a transpeptidase domain, and a transglycosylase domain needed for the proper construction of bacterial cell wall (Thomas et al., 1992). The role of other PBPs in conferring β-lactam resistance to *H. pylori* has not been studied extensively and is yet to be established. In other microorganisms PBPs such as PBP2 have been implicated as playing a role in β-lactam resistance (Rosenstock et al 2003; Haenni et al., 2006)

Gram negative bacteria have porin proteins in their outer membrane which facilitate the transport of hydrophilic solutes. In previously obtained clinically resistant strains, changes in PBP1 alone do not account for all of the resistance observed. In addition to changes in PBPs, reduced membrane permeability and active efflux have been suggested as mechanisms contributing to higher resistance to β-lactam drugs. In two studies, this has been tested with the proton translocator inhibitor CCCP. Amoxicillin resistant *H. pylori* strains accumulated less [^{14}C]-penicillin G compared with amoxicillin-susceptible strains, both in the presence and absence of CCCP, thus excluding the role of an active efflux mechanism (DeLoney et al., 2000; Kwon et al., 2003). This suggests that at least in some strains β-lactam resistance in *H. pylori* is partly due to an increased diffusional barrier, an effect that could be explained by changes in outer membrane proteins. By comparing an in vitro selected amoxicillin resistant strain with its amoxicillin sensitive parent strain it has been determined that both changes in PBP1 and Hop Proteins B and C are important for conferring β-lactam resistance (Co et al., 2006).

Objectives

To better understand the mechanisms of β-lactam resistance in *H. pylori*, a number of approaches were used in this study. Furthermore, both a clinically amoxicillin resistant isolate and in vitro derived amoxicillin resistant strains were characterized.

Initial studies were done to determine the effect of previously published single amino acid mutations and the resistance they confer to *H. pylori*, along with the effect combined mutations and determining whether this effect is additive. Homology modeling studies were done to place these mutations in the three dimensional space of PBP1 to visualize and hypothesize how their interactions in that space result in conferring resistance. Putative Penicillin Binding Motifs (PBMs) were also modeled to view their relative location in the protein and to each other.

Further studies were done to characterize the clinically resistant isolate B258 of *H. pylori*. We believe this is the first United States amoxicillin resistant clinical strain to be characterized. *Pbp1* from B258 was PCR amplified and transformed into an amoxicillin sensitive strain 26695 and shown to account for all the resistance seen in B258. Its sequence showed 13 amino acid changes compared to wild type PBP1 and these changes were systematically transformed into 26695 to determine which changes were responsible for this resistance.

Finally an in vitro resistant *H. pylori* strain was created by consistently growing and passing the amoxicillin sensitive 26695 strain on increasing levels of amoxicillin. Over a period of 9 months the strain became about 90 fold more resistant to amoxicillin. Initial sequencing of some potentially altered genes only showed a couple of amino acid

changes. Whole genome sequencing was employed to locate other changes that occurred. Twelve mutations were found in various genes. Strains with intermediate levels of amoxicillin resistance which emerged during this in vitro selection experiment were collected and studied. These genes were also sequenced in these intermediate amoxicillin resistant isolates to determine the evolution of resistance.

The ultimate goals of these studies are to:

- identify the minimum number of amino acid changes in PBP1 which can confer amoxicillin resistance
- Localize these sites in PBP1 using homology modeling
- Explore the possibility of additional amoxicillin resistance mechanisms in both a clinical isolate and in vitro isolates.

Chapter 2

Characterization of Frequently Occurring Mutations in Penicillin Binding Protein 1(PBP1)

Introduction

There are 3 major PBPs that have been identified in *H. pylori* (PBP1, PBP2, PBP3); PBP4 is a minor PBP and its role in peptidoglycan synthesis is yet to be determined. PBP1 has been most extensively studied and has been shown to play a significant role in conferring β-lactam resistance to *H. pylori* (Co et al., 2006; Kwon et al., 2003; Gerrits et al., 2002 and 2006; Matteo et al., 2008; Okamoto et al., 2002; Paul et al., 2001 and Rimbara et al. 2007). The identity of various amino acid changes seen in PBP1 by these investigators is summarized in Table 1. In a few cases single amino acid mutations, such as T438M (Co et al., 2006) and S414R (Gerrits et al., 2002), appear to be sufficient to cause increased amoxicillin resistance. We have also demonstrated that the single S414R substitution in PBP1 causes a 2X increase in resistance to amoxicillin (unpublished) [Note: The role of T556S in amoxicillin resistance, as reported by Matteo at al., 2008, was not known when we did the experiment described below].

	S402G	E406A	S414R	S417T	T438M	Y484C	E506A	M515I	S517T	D535N	T541I	S543R	T556S	N562Y	T593A	P600T
Co					X											
Gerrits			X													
Gerrits	X	X	X	X												
Kwon							X	X	X	X		X	X	X		
Matteo													X			
Okamoto													X	X	X	
Paul			X			X					X		X	X		X
Rimbara			X											X		

Table1. Review of previously identified mutations in PBP1 from amoxicillin resistant isolate. Nothe the high incidence of certain mutations in these strains.

On the other hand, most studies have reported multiple amino acid changes in PBP1 without determining the importance of individual mutations. Mutations in PBP1 such as S543R, T556S and N562Y have been associated with resistant *H. pylori* strains along with several other mutations (Kwon et al., 2003; Matteo et al., 2008; Okamoto et al., 2002; Paul et al., 2001 and Rimbara et al., 2007). The contribution of these individual mutations to amoxicillin resistance is not clear since several other mutations are present in PBP1.

In this study we used site-directed mutagenesis to create these three mutations individually in PBP1 and then transformed each altered PBP1 into strain 26695 (amoxicillin sensitive) to monitor its effect on the Minimum Inhibitory Concentration (MIC) of *H. pylori* 26695 to amoxicillin. Next we wanted to identify whether individual mutations conferring resistance, when combined together would have a cumulative effect on the MIC. These studies showed that the S543R, T556S and N562Y individually increase resistance in *H. pylori*, and in combination the effect of these mutations is cumulative.

Materials and Methods

Strains and culture conditions

Strain 26695 was used as a reference strain and for creating transformants. Stock cultures of *H. pylori* 26695 were streaked for isolation on Brucella Agar (Becton-Dickinson Microbiology, Cockeysville, MD) supplemented with 5% defibrinated sheep blood (Colorado Serum Company, Denver, CO) and cultured at 37°C in a humidified

10% CO_2 incubator. Liquid cultures were prepared by suspension of *H. pylori* colonies in Brucella broth (Difco Laboratories, Detroit, MI) supplemented with 10% heat-inactivated fetal bovine serum (Gibco Bethesda Research Laboratories, Grand Island, N.Y.) and 1% IsoVitaleX (Becton-Dickinson Microbiology). Cultures were routinely passed by dilution into fresh media at 48 h intervals. Freezer stocks of cultures were prepared by re-suspending 48 h cultures in 1% proteose peptone-20% glycerol, flash frozen in liquid nitrogen, and kept at -80°C.

General DNA Techniques

All DNA manipulations were performed according to standard protocols. Primers used in this study were created using the published sequence of *pbp1* of 26695 and Vector NTI (Invitrogen, Carlsbad, CA) and synthesized by Sigma-Aldrich (The Woodlands, TX). Polymerase chain reaction (PCR) was performed in an automated thermal cycler PTC100 (MJ Research, Ramsey, MN), using the Choice Taq Blue Polymerase (Denville Scientific, Metuchen, NJ). PCR products were run on 1% agarose gels (Biorad, Hercules, CA) and subsequently cut and purified using Minelute agarose purification system (Qiagen Inc., Valencia, CA). Sequencing of purified DNA product was performed by Genomics Core Institute at the University of California Riverside.

Site Directed Mutagenesis

The PBP1 gene from 26695 was cloned into a pUC19 (New England Biolabs) vector and used for creating site-directed mutations using the Quick-change Site-Directed

Mutagenesis kit (Stratagene). Mutagenic primers were generated based on the desired change and specifications of the kit. The Quick-change Site-Directed Mutagenesis system uses a PCR-based procedure that incorporates the desired mutation into the plasmid, generating a plasmid with modified *pbp1*. The PCR was performed according to the conditions in the system protocol. The product was then transformed into XL1-Blue supercompetent cells (Stratagene) and plasmid was purified using Miniprep from a few ampicillin resistant colonies and sequenced to confirm the presence of mutation. Dual mutations were created by repeating this procedure for each mutation. The mutated *pbp1* was then amplified off the plasmid using primers shown in table 2, and products were run on 1% agarose gel with 0.5µg/ml ethidium bromide and isolated using Minelute gel extraction kit. The product was quantified using Beckman DU 530 UV/VIS spectrophotometer and 100-150 ng was used to transform 26695 by the procedure of Wang et al., 1993 using ECM 630 Electroporator (BTX, Hawthorne, NY). The transformants were plated on Brucella-blood agar plates. After 48 hours the bacteria were transferred on Brucella-blood agar plates with amoxicillin with concentrations of 0.125, 0.25 or 0.5 µg/ml. All culture conditions are described below. Resistant colonies were isolated and their genomic DNA purified using DNeasy tissue kit (Qiagen). PBP1 was PCR amplified and sequenced to confirm the presence of desired mutation.

Primer Name	Primer Sequence
HP-PBP-2L	5’-GTTCCTCGCTATCGTCTGTTCTTTTGG-3’
HP-PBP-2R	5’-CGTTTTGATTGTGATGGGGTTATTA-3’

Table 2. Nucleic acid sequence of primers used to amplify the PBP1 gene from genomic DNA of *H. pylori*

Minimum Inhibitory Concentration (MIC) protocol

The MIC of amoxicillin was determined for all strains as follows. Serial fold dilutions of amoxicillin (Sigma) were prepared in supplemented Brucella Broth in a 96 well plate (Corning). Starting with 150 µl of 16 µg/ml amoxicillin in the first well, amoxicillin was diluted to 8, 4, 2, 1, .5, .25, .125, .06, .03, .0125 and .006 µg/ml in the subsequent wells. *H. pylori*, with an inoculum OD600 (optical density at 600nm) of 0.5±.05 were inoculated at a 1:100 dilution (1.5µl in 150µl, ~ 10^6 cells). The 96 well plates were grown at 37°C, with 10% CO_2 and 100% humidity. Plates were allowed to incubate for 3 days and wells were examined for visible signs of growth. MIC was determined to be the lowest concentration of amoxicillin that could inhibit the growth of bacteria. The parental 26695 strain was used as a control in all MIC determinations. Positive (no antibiotic) and negative (no bacteria) controls were included on each plate for each strain being tested.

Amoxicillin affinity measurements on 26695 mutant strains

Biotinylated amoxicillin (Bio-Amox) was prepared as described by Dargis and Malouin (1994). Membrane preparations of sensitive and mutated strains were obtained by growing cells on 10 Brucella blood agar plates for 2-3 days at 37°C, with 10% CO_2 and 100% humidity. Bacterial colonies were swabbed into 20 ml of 0.1M sodium phosphate buffer, centrifuged for 20 min at 10,000 RPM, washed twice and resuspended to a final volume of 10 ml. Cells were frozen overnight to help cell disruption. Cells were thawed the next day and 1mg/ml of lysozyme was added and incubated on ice for 20 min.

Afterwards, cells were disrupted using a Vibra Cell sonicator (Sonics and Materials Inc.) and spun down at 10,000 RPM for 30 min to remove unbroken cellular components. Membrane fractions in the supernatant were collected by ultracentrifugation using a Beckman L8-70M ultracentrifuge (Beckman) at 28,900 RPM.

Protein concentration in the membrane fractions was determined using Protein assay reagent (Biorad) with IgG as standard (Biorad). Membrane fractions (20 µg of protein) were labeled with final concentrations of Bio-Amox at 2.0, 1.0, 0.8, 0.4, 0.2, 0.1, .05, .02 µg/ml for 30 min at room temperature. Samples were boiled for 5 min for further cell disruption and run on a 14% SDS-polyacrylamide gel for separation. Proteins were subsequently transferred to a nitrocellulose membrane (Amersham) using a mini Trans-blot cell (Bio-Rad). The membranes were blocked overnight with 5% blocking reagent in PBS-Tween, and then treated with streptavidin conjugate (Amersham) to allow visualization on an ECL hyperfilm (Amersham). Density measurements to compare absorbance intensities of the resulting bands were performed on the film using Odyssey software (Licor).

Alternatively, affinity assays were also done with whole cell preparations. With this method, 1ml of 48 h culture was spun down and resuspended in 100µl. Cell preparation (contains 100 µg of protein) aliquots were labeled with final concentrations of bio-Amox at 2.0, 1.0, 0.8, 0.4, 0.2, 0.1, .05, .02 µg/ml for 30 min. Subsequent treatments were same as procedures performed on the membrane protein preparations.

Results and Discussion

Previous studies in our lab have already identified a few amino acid changes in PBP1 which directly affect amoxicillin binding to PBP1 (S414R, T438M). Using site-directed mutagenesis, seven additional PBP1 transformants were constructed; 4 of these are PBP1 with single amino acid substitution while 3 are double mutants (see Table 3). Single amino acid mutations close to penicillin binding motifs (PBMs) (See Fig. 1), such as S543R, T556S and N562Y, resulted in modest changes in MIC ranging from 0.125-0.25 µg/ml compared to the original value of .06 µg/ml. Single mutations S543R, T556S and N562Y resulted in an increase in resistance proportional to their distance from the PBMs.

PBP1 Transformants	MIC value (µg/ml)
26695 (reference strain)	.06
S543R	.125
T556S	.25
N562Y	.25
T511V	.06
N562Y+S543R	.5
N562Y+T556S	1
S543R+T556S	.5

Table 3. MIC values for transformants. The mutant strains are denoted by the symbol of the original amino acid, the locus of the mutation and the symbol of the changed amino acid. Representative of atleast three replicates of this experiment.

S543R, which is further away from the PBM at 555-557 resulted in an MIC value of 0.125 µg/ml, whereas T556S and N562Y which are both within PBMs, resulted in an MIC value of 0.25 µg/ml (Fig. 1) One of the single mutants, T511V did not confer

significant resistance, probably since it is not located near any PBM. Since the selection process for screening the mutants depends on their increase in resistance, it is likely that T511V confers less than a 2X increase in resistance, perhaps 1.5X, just enough to isolate it. Aside from being localized in the transpeptidase domain, the mutations conferring significant resistance are localized mostly around the Penicillin Binding Motifs, particularly around the KTG position starting at 555th residue and SKN position starting at 403 residue. Mutations not localized around these motifs did not result in an amoxicillin resistant strain and hence were not detected (Fig. 1).

As hypothesized, the double mutants resulted in additive resistance (See Table 3). The two single mutations N562Y and T556S, each of which resulted in an increase in MIC value of 0.25 µg/ml individually, together resulted in an MIC value of 1 µg/ml. S543R (MIC 0.125 µg/ml) when combined with either N562Y or T556S (MIC 0.25 µg/ml) resulted in an increase in MIC to a value of 0.5 µg/ml.

While some investigators have suggested the importance of amino acid change in PBP1 to amoxicillin resistance, few have documented the actual effect on amoxicillin binding. In this study, using bio-Amox, the binding affinity of amoxicillin and PBP1 from the sensitive strain and PBP1 containing single or double mutation was determined. As shown in Fig. 2, density readings showed that Bio-Amox saturation of PBP1 in 26695 occurs with as little as 0.4 µg/ml Bio-Amox but in the double mutant, with S543R and T556, requires 2 µg/ml bio-Amox (see Fig. 2). Binding affinity experiments on all single and double mutants showed a change in affinity comparable to a change in MIC (data not shown) and a pattern similar to that shown in Fig. 2. The increase in level of MIC due to

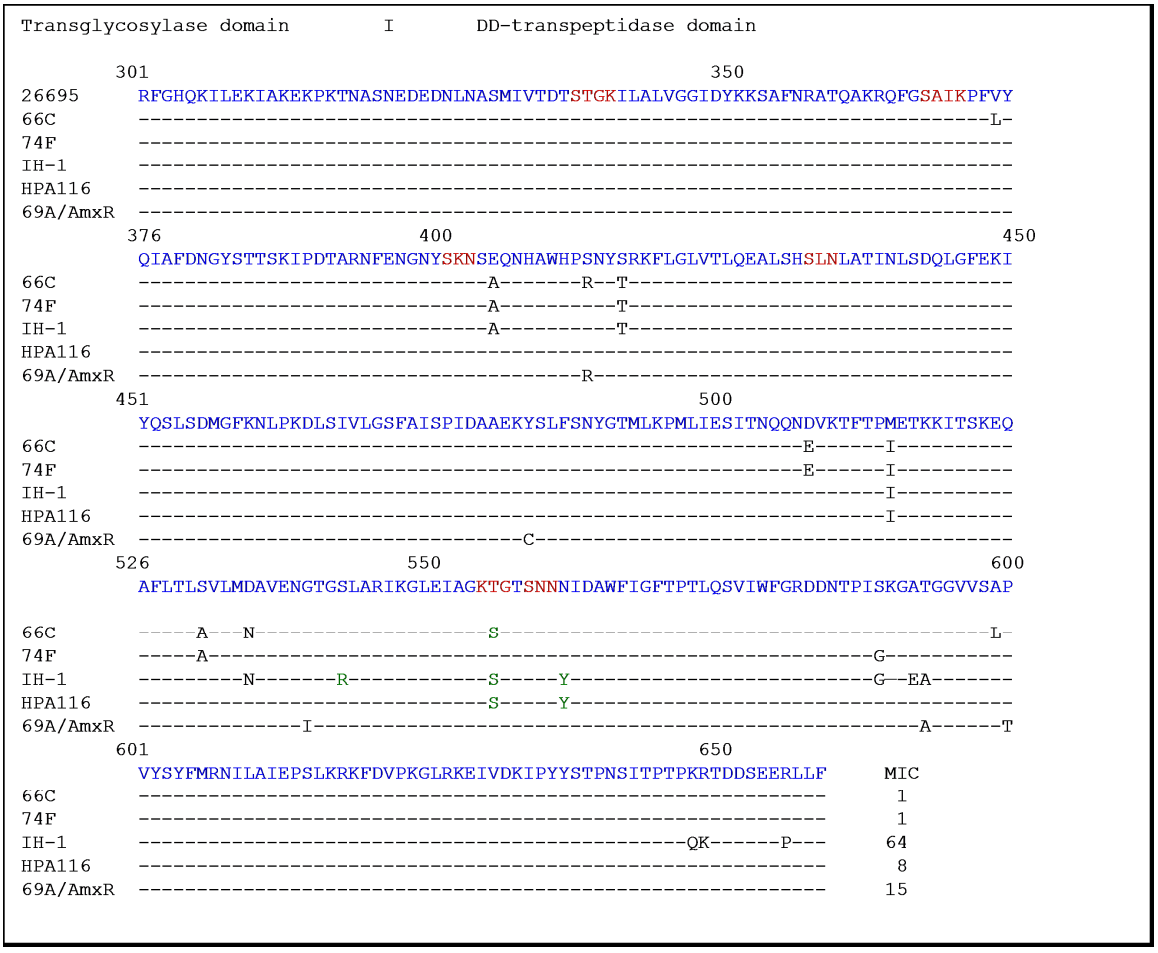

Fig. 1. Alignment of PBP 1 transpeptidase regions from 26695 and several amoxicillin resistant strains showing amino acid differences. The PBMs are shown in red, whereas the single and double amino acid mutations used in this study are shown in green.

point mutations in PBP1 is inversely proportional to the decrease in binding affinity of PBP1 to Bio-Amox.

These results show that certain mutations in or around the PBMs in the transpeptidase domain of PBP1 result in increased resistance to β-lactams by decreasing their PBP1 binding affinity. It also shows that individual mutations conferring resistance when combined have an additive effect on increase in resistance.

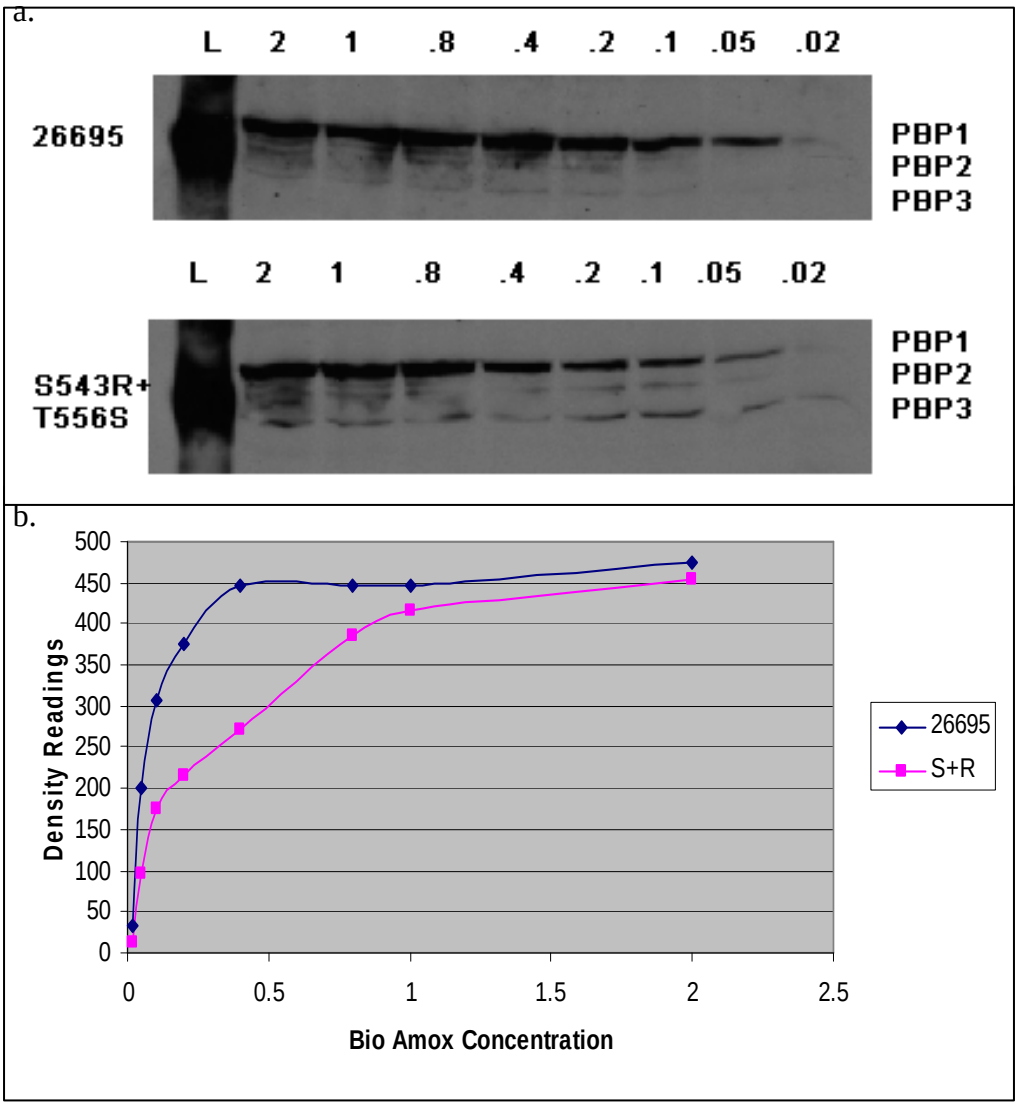

Fig. 2. Bio-Amox affinity assay: a) Western blot showing binding of bio-Amox to whole cells from reference strain 26695 and double mutant S543R+T556S; b) Density reading plot against bio-Amox concentration.

This study focused on 3 specific amino acid mutations in PBP1, however several other mutations have been observed in various resistant strains (Table 1 and Fig. 1). Studying all mutations found in these strains can be challenging. Proximity to PBMs has been used to suggest whether a mutation might result in increased resistance. However

the role of PBMs has not been established or suggested beyond speculation. To make the process of assessing the likelihood of these changes contributing to an increase in resistance, we wanted to use a more systematic approach. Homology modeling is used to visualize the 3-dimensional structure of a protein. Creation of a homology model of PBP1 would allow us to better visualize the relative position of each amino acid as well as allow us to better predict the role of various mutations.

Chapter 3:

3-Dimensional Modeling of PBP1 from *H. pylori* and Putative Penicillin Binding Motifs (PBMs)

Introduction

Individual mutations in PBP1 so far have only been shown to contribute a moderate, but significant increase in amoxicillin resistance. In other organisms individual mutations have been shown to be responsible for a higher level of resistance. Previously six putative PBMs have been identified in *H. pylori* PBP1 and proximity to these PBMs has been used to predict whether a mutation is likely to confer amoxicillin resistance. This criteria does not take into account the relative 3-dimensional location of the amino acids to each other or to the catalytic amino acid/s. Only a handful of mutations in amoxicillin resistant isolates have been characterized individually, and the role of the remainder of the mutations has yet to be determined. Since it is only possible to isolate transformants with mutations conferring amoxicillin resistance owing to the nature of the selection process, it is difficult to systematically hypothesize on the role of multiple mutations.

We hypothesized that by looking at the three dimensional structure of PBP1, better predictions could be made as to the amino acids which are most likely involved in the interaction between amoxicillin and PBP1. A protein model could also be used to identify those specific mutations seen in clinical strains which are most likely to explain the amoxicillin resistance reported. Homology modeling uses the three dimensional structure of proteins determined by either NMR or X-ray crystallography to predict the structure of another homologous protein. Generally each domain of a protein is modeled individually. Sequences are submitted to Swiss-Model which is a protein structure

homology modeling server accessible via the Expasy web server. The proteins can be modeled using the best template available or a specific template can be specified.

The *H. pylori* PBP1 consists of the transglycosylase domain and the transpeptidase domain. The transpeptidase domain of PBP1 is thought to be responsible for binding amoxicillin and this domain was used for modeling studies. Molecular surface and electrostatic potential was also determined. The model showed the putative catalytic cleft, and the relative location of various amino acids and PBMs.

Materials and Methods

Homology Modelling

With the guidance of Dr. Dimitrios Morikis from the department of Bioengineering, the three dimensional structure of the transpeptidase domain of PBP1 was created using homology modeling. The location of the transglycosylase domain and transpeptidase domain was determined by a protein domain identifier site, Pfam. The transpeptidase domain starts around amino acid 320. The sequence from 320-659 was screened against PBP1 sequences in the RCSB Protein Data Bank in order to identify a template structure appropriate for modeling. We selected PBP1A from *Streptococcus pneumoniae* 2c6Wb as it had the highest identity (26%) and similarity (38%) to the transpeptidase domain of *H. pylori*. The structure of *S. pneumoniae* PBP1A is available at 2.6 Å (Windle et al., 2000). It also contains a transpeptidase domain and a transglycosylase domain. The truncated sequence was then submitted to Swiss-Model using the first mode approach and the *S. pneumoniae* molecule found earlier was used as

a template. The sequence PDB files were received via email and then viewed in SPDV-Deep View (Bioscience IT). The PBP1A template from *S. pneumoniae* was viewed while overlapping the homology model of PBP1 from *H. pylori* (Fig. 3).

Model Analysis

Several different analyses were done to determine the overall properties of the molecule. Using SPDV-Deep View the secondary structure of the protein, hydrophobicity and solvent accessibility was determined. Hydrogen bond patterns were located along with calculation of molecular surface, and electrostatic potential. To determine the location of PBMs, the model was visualized as a ribbon (Fig. 4). PBM side chains were made visible in green whereas all other side chains were made invisible (Fig. 4). Using *S. pneumoniae* PBP1A as a model for possible interactions in *H. pylori* PBP1, the potential active site was identified. Hydrogen bonding patterns between conserved PBM amino acids were analyzed.

Results and discussion

The PBP1 from *H. pylori* amoxicillin sensitive strain 26695 was modeled against the crystal structure of PBP1A from *S. pneumoniae*. Sequence identity between the two proteins of 26% is not as high as would be ideal, however it is above the acceptable 20% value and the sequence similarity of 38% makes it favorable. The model of PBP1 from *H. pylori* when superimposed with the template from *S. pneumoniae* shows high conformity with some differences within the loops. (Fig. 3). The model shows several small clefts as

well as one major cleft, likely to be the putative catalytic cleft. PBP1 mutant isolates from various sources (Table 1 and Fig. 4a) show a pattern of recurring mutations, suggesting a unique PBP1 drug resistance mechanism likely to have some connection to the putative binding cleft.

Fig 3. Ribbon representation of crystal structure of PBP1 A from *S. pneumoniae* is shown in blue, and compared to ribbon representation of homology model of PBP1 from *H. pylori* shown in yellow.

4a.

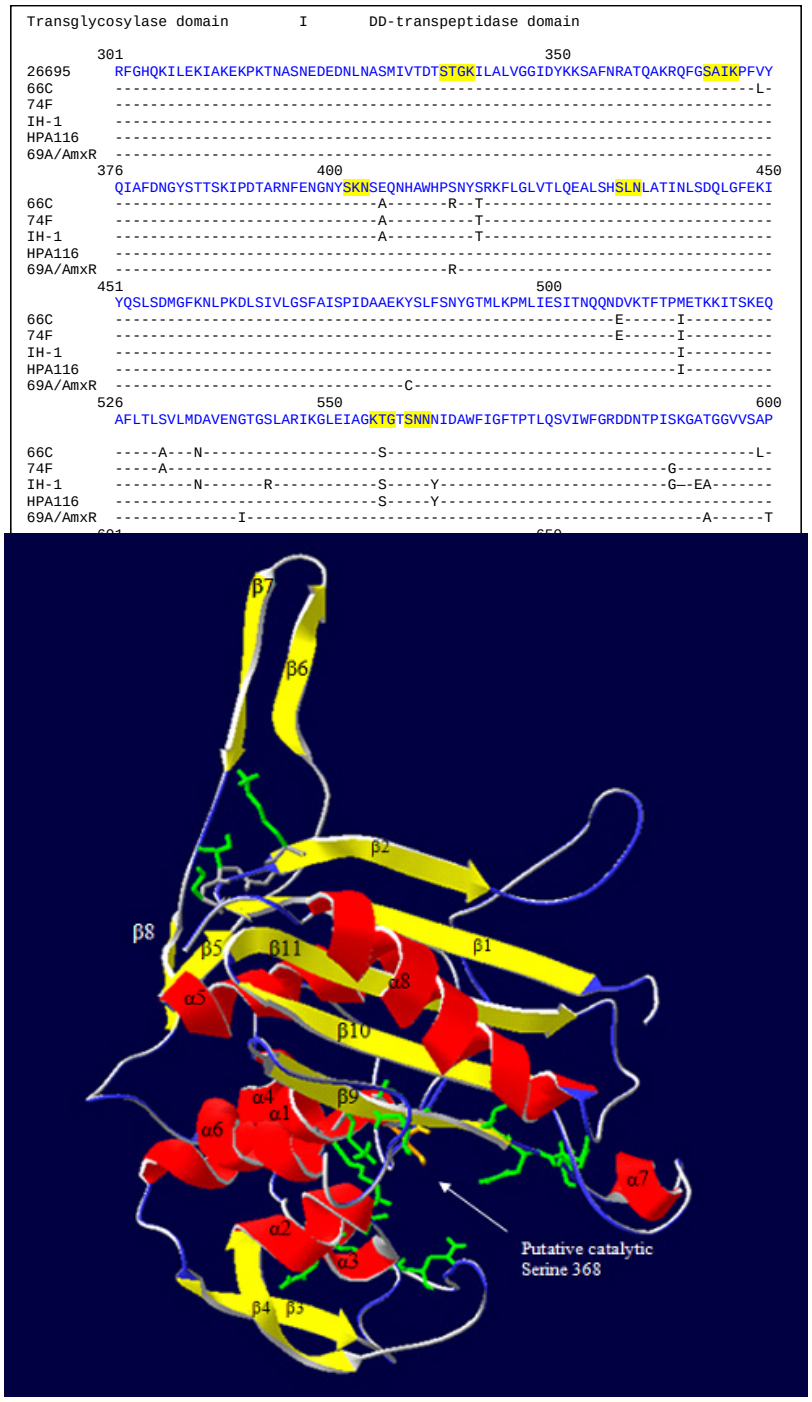

Fig. 4a. Sequence of PBP1 with the PBMs highlighted in yellow. 4b. Three dimensional model of PBP1 from *H. pylori*. Conserved penicillin binding motif side chains are in green. All other side chains are not shown. Putative catalytic serine 368 side chain is shown in orange.

The transpeptidase domain of PBP1 consists of about an equal number of alpha helices and beta sheets. Two β-sheets within the domain are part of the linker region (β6, β7). The transpeptidase domain starts with β-sheets (β1, β2), and displays substantial similarity with other proteins that bind and /or cleave β-lactam antibiotics, such as β-lactamases, transpeptidases and other PBPs. Albeit the low level of sequence identity between these macromolecules, a central, five-stranded β-sheet (β1, β2, β9, β10, β11) is highly conserved. α7 and α8 are present in front of the sheets whereas α1- α6 are present toward the back. The putative active site is embedded deep within the putative catalytic cleft and is surrounded by β9, α1, α2 and α3 (Fig. 4b.).

H. pylori PBP1 is thought to have six conserved PBMs in the transpeptidase domain. As seen in Fig. 4b, five of these motifs fall within the putative binding cleft with their side chains accessible through the cleft. The first conserved sequence motif consisting of Ser 338, Thr 339, Gly 340 and Lys 341 in transpeptidase domain is between two β-sheets (β1 and β2). The location of this PBM block places it in the linker region between the transpeptidase and transglycosylase domain. This conserved motif is considered part of the six PBMs, however its location does not suggest a direct role in penicillin binding but it is more likely to be crucial for a stable interaction of the two domains. α1 is the first helix of the transpeptidase domain. The second PBM contains the putative catalytic Serine 368 and consists of Ser 368, Ala 369, Ile 370 and Lys 371. The third conserved PBM in *H. pylori* PBP1 is Ser 403, Lys 404, Asn 405. The fourth PBD consisting of Ser 434, Leu 435 and Asn 436 follows the conserved motif of SXN and its

location is homologous to that in *S. pneumoniae* (Contrares-Martel et al., 2006). Lys 555, Thr 556 and Gly 557 show a conserved motif KTG.

The SXXK, SXN and KTG motifs in *H. pylori* PBP1 are also conserved in PBPs in other organisms as well (Ghuysen et al., 1996; Spratt et al., 1998). This is immediately followed by Ser 559, Asn 560 and Asn 561 which is only shown to be conserved in *H. pylori* (Fig. 4a).

The protein has an overall positive charge of +5. The electrostatic surface representation of PBP1 shows a majority of positive charges but negative charges in small isolated regions of the protein are apparent. The putative binding cleft shows both a positive and a negative charge (Fig. 5). Changes in the charge distribution of the putative binding cleft could potentially alter its affinity for β-lactams, resulting in resistance to the drug.

The crystal structure of PBP1A from *S. pneumoniae* contains a catalytic cleft housing Ser 370 which is the catalytic serine. The catalytic serine is essential for the function of the protein and also covalently binds to β-lactams rendering it incapable of performing its transpeptidase function (Contrares-Martel et al., 2006) The homology model of PBP1 from *H. pylori* also possesses a similar putative catalytic cleft and putative catalytic serine at 368. Pka value determinations showed Lys 371 and Lys 555 with a much lower Pka then expected within 3.5A of the putative catalytic Ser 368, suggesting the presence of an active site. Interactions of the putative catalytic serine with its environment are discussed further in the next chapter.

Fig 5. Electrostatic surface representation of homology model of PBP1 from *H. pylori*. Blue indicates positive charge, whereas red indicates negative charge.

In the unliganded form a surface representation of PBP1 (Fig. 6) reveals that two residues Tyr 416 and Asn 560 extend into the putative binding cleft which likely limits entry into the tunnel-like catalytic gorge. This interaction may present a stabilizing effect in the absence of a ligand. The close proximity of the interactions between Tyr 416 and Asn 560 suggest that prior to catalysis, natural substrates must be threaded into the active site in order to reach Ser 368 (Contrares-Martel et al., 2006).

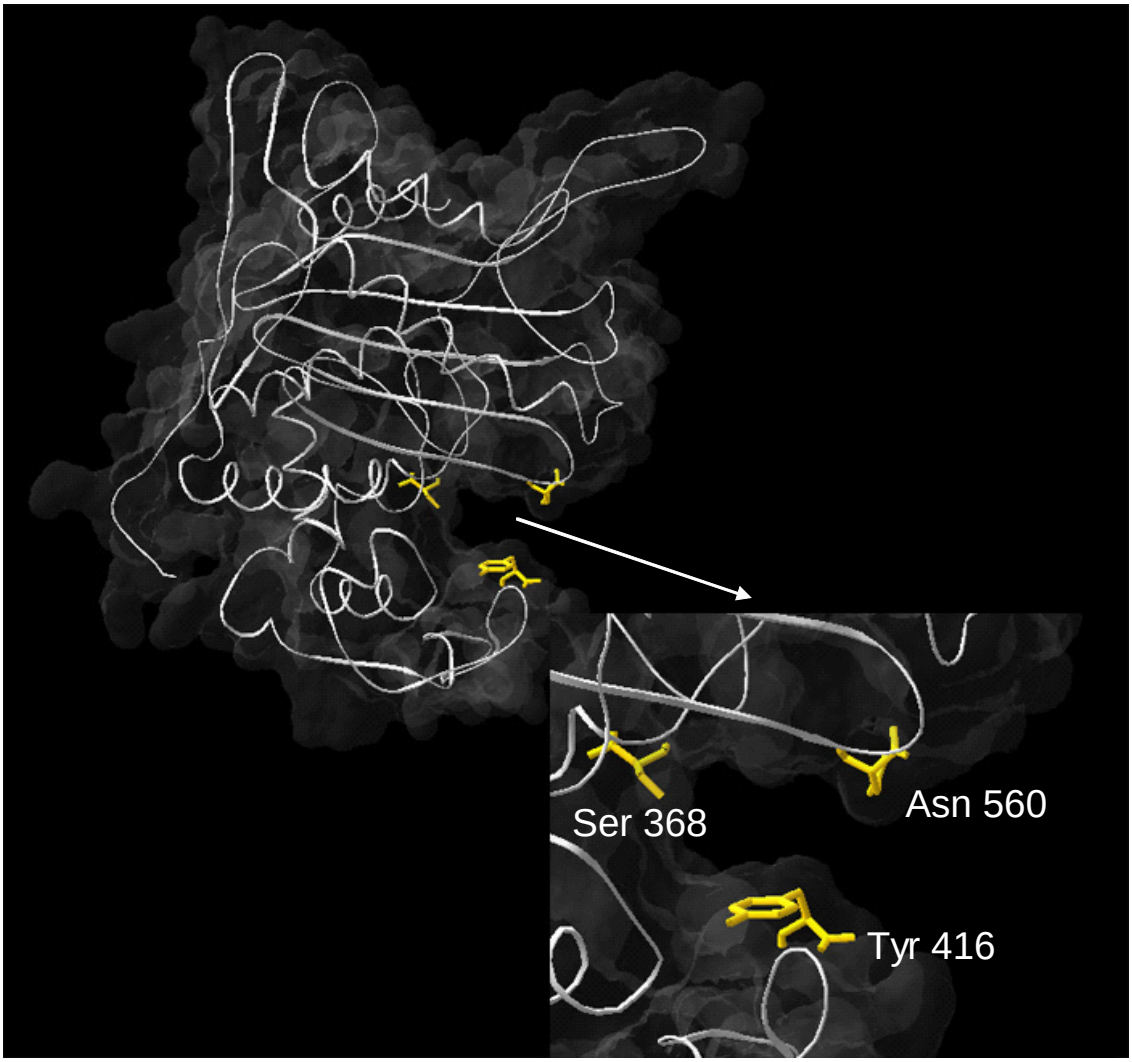

Fig 6. Molecular surface representation of the homology model of PBP1 from *H. pylori*. Asn 560 and Tyr 416 are protruding into the entrance of the putative binding cleft.

This study has given insight into the structure of PBP1 protein from *H. pylori*. Many resistant strains show several mutations and determining the contribution of most mutations to amoxicillin resistance could only be speculated. One method used to determine if a mutation is responsible is to transform it into an amoxicillin sensitive

strain. However if a mutation has a minor effect or no effect, it cannot be isolated using antibiotic selection. Using the 3-dimensional homology modeling, better predictions can be made for the role of individual mutations in amoxicillin resistance.

Of the 6 previously established PBMs only five were found in the putative binding cleft. The first PBM is conserved not likely for its role in penicillin binding but more likely for the interaction of transglycosylase domain with the transpeptidase domain. Previously identified mutations occurring in PBP1 of amoxicillin resistant strains of *H. pylori* were assumed to be important based on their proximity to the PBMs, but positioning of these PBMs within the protein had never been established. It is possible that a mutation not close to a PBM could be in proximity to the putative binding cleft and/or putative catalytic serine. The environment within the cleft is likely crucial for substrate and β-lactam binding resulting in conservation of these five PBMs. Mutations altering the amino acids in this environment probably result in either blocking access to the putative catalytic serine or cause inefficient binding.

Further studies were performed by combining molecular biology experiments with homology modeling. Homology modeling has its limitations and cannot be substituted for a crystal structure. However it can be used to make better predictions and hypotheses. In the next chapter, a homology model of PBP1 from an amoxicillin sensitive strain and an amoxicillin resistant strain from *H. pylori* were compared. Interactions of mutated amino acids from the resistant strains were compared to amino acids found in amoxicillin sensitive strains. Analysis of previously occurring mutations in amoxicillin resistant strains showed a strong correlation between mutations present in the cleft and

amoxicillin resistance. Identification of mutational hotspots in *H. pylori* is important as it allows for the development of molecular techniques that will rapidly detect for resistance mechanisms in clinical isolates. This model could provide us with valuable information for the development of novel antibiotics for the treatment of β-lactam resistant *H. pylori* strains.

Chapter 4:

Contribution of Specific Amino Acid Changes in Penicillin Binding Protein-1 to Amoxicillin Resistance in Clinical *Helicobacter pylori*

Introduction

Helicobacter pylori infection is strongly associated with the development of numerous gastric pathologies, including chronic type B gastritis, gastric and duodenal ulcers, gastric adenocarcinoma and B-cell mucosa-associated lymphoid tissue (MALT) lymphoma (Dunn et al., 1997). It is the most common cause of peptic ulcer disease (Rosenstock et al., 2003) and is responsible for 5.5% cases of human cancer each year (Parkin et al., 2006). There is also some evidence to suggest that *H. pylori* may infect other organs including the liver (Dunn et al., 1997), and may be associated with Alzheimer's disease (Malaguarnera et al., 2004). Currently, the rate of *H. pylori* infection in middle-aged adults is 20-50% in industrialized countries and 80% or more in many developing countries (Frenck et al., 2003; Rothenbacher et al., 2003), with higher prevalence rates seen in people of low socioeconomic status (Frenck et al., 2003).

Effective treatment of *H. pylori* infection typically requires a combination including two antibiotics and a proton pump inhibitor or a bismuth compound (Gerrits et al., 2006). First line antibiotics include amoxicillin, clarithromycin, tetracycline and metronidazole (Gerrits et al., 2006). While often effective, as many as 20 to 30% of these treatments fail, usually as a result of a lack of patient compliance or increasingly to antibiotic resistance (Gerrits et al., 2006).

Antibiotic resistance of *H. pylori* to many of these antibiotics is increasing, with various levels of resistance noted (Megraud et al., 2005). The rate of resistance is higher in developing countries as compared to that in developed countries mainly due to the variation of prescribing patterns (Hu et al., 2007). Many strains of *H. pylori* are naturally

competent allowing it to take up resistance genes from other microbes as well as naked DNA from the environment (Nadenskov-Sorensen et al., 1990; Tsuda et al., 1993). However most of the antibiotic resistance mechanisms described to date are a result of point mutations on the bacterial chromosome (Gerrits et al., 2006), a consequence of a significantly high mutation rate in *H. pylori* (Megraud et al., 2007).

Although the prevalence rates of amoxicillin resistance in *H. pylori* strains in the United States are generally very low, resistance rates as high as 33% have been seen in other countries (Hu et al., 2007). Resistance to β-lactam drugs in Gram-negative bacteria is mostly due to β-lactamase activity (Bush 2001). Although the *H. pylori* genome contains a β-lactamase-like gene which codes for the protein HcpA (Mittl et al., 2000), this does not appear to confer amoxicillin resistance. Tseng et al., (2009) recently described the presence of a TEM β-lactamase conveying high level amoxicillin resistance in a clinical isolate from Taiwan; however amoxicillin resistance in most clinical isolates of *H. pylori* is not due to β-lactamase activity. For these strains resistance seems to be mainly mediated by alterations to penicillin-binding proteins (PBPs). The role of amino acid mutations in PBP1 in amoxicillin resistance has been described in a number of studies, both in clinical isolates (Kwon et al., 2003; Gerrits et al., 2002 and 2006; Matteo et al., 2008; Okamoto et al., 2002 and Rimbara et al. 2007) as well as in vitro derived amoxicillin resistant strains (Co et al., 2006, Paul et al., 2001). In some cases, this moderate to low-level amoxicillin resistance has been attributed to single amino acid substitutions (Co et al., 2006, Gerrits et al., 2002, Matteo et al., 2008), whereas others

have reported multiple amino acid changes (Kwon et al., 2003; Matteo et al., 2008; Okamoto et al., 2002 and Rimbara et al. 2007).

In this report we have characterized the amoxicillin resistance of a clinical isolate identified in the US (to our knowledge, the first US strain to be characterized), identified the role of amino acid substitutions in PBP1 in resistance, and used homology modeling to clarify the role of these substitutions in affecting amoxicillin binding. We have also examined the previously published amino acid substitutions with structural and physicochemical analysis of the homology models in order to better understand their contributions to amoxicillin resistance. This approach has led to the hypothesis of a defined set of amino acid substitutions in PBP1 which affect amoxicillin binding and consequently reduces the sensitivity of these strains to the bactericidal effect of amoxicillin.

Materials and Methods

Bacterial strains and culture conditions

The *H. pylori* clinical isolate B258 was kindly provided by Dr. Richard M. Peek, Jr., Department of Gastroenterology, Vanderbilt University Medical Center. *H. pylori* ATCC strain 26695 was used as a reference strain and for creating transformants. Stock cultures of *H. pylori* B258 and 26695 were streaked for isolation on Brucella agar supplemented with 5% defibrinated sheep blood and cultured at 37°C in a humidified 10% CO_2 incubator. Liquid cultures were prepared by suspension of *H. pylori* colonies in Brucella broth supplemented with 10% heat-inactivated fetal bovine serum and 1%

IsoVitaleX. Cultures were routinely passed by dilution into fresh media at 48 h intervals. Freezer stocks of cultures were prepared by re-suspending 48 h cultures in 1% proteose peptone-20% glycerol, flash frozen in liquid nitrogen, and kept at -80°C.

General DNA Techniques

All DNA manipulations were performed according to standard protocols. Primers used in this study were created using the published sequence of *pbp1* of 26695 and Vector NTI and synthesized by Sigma-Aldrich. Polymerase chain reaction was performed in an automated thermal cycler PTC100, using the Choice Taq Blue Polymerase. PCR products were run on 1% agarose gel and subsequently cut and purified using Minelute agarose purification system. Sequencing of purified DNA products was performed by the Genomics Core facility at the University of California, Riverside.

Transformation of *H. pylori*

Transformations of the 26695 reference strain were done using ~200 ng of purified PCR amplified DNA and the electroporation protocol described by Wang et al., 2003 using ECM 630 Electroporator. Transformation mixtures were plated on Brucella agar supplemented with 5% defibrinated sheep blood; after 48-72 h, transformants were selected on Brucella agar medium supplemented with 5% defibrinated sheep blood and 0.125 µg/ml amoxicillin.

Generation of 26695-T and other transformants

The transpeptidase region of *pbp1* from B258 was PCR amplified, purified and subsequently used to transform strain 26695. Various fragments of the transpeptidase domain from B258 (as shown in Figure 8c) were PCR amplified, purified, and used to transform 26695. Site directed mutations were created for the Ser 543 Arg and Met 533 Ile loci using the Quick Change Site-Directed Mutagenesis kit. Potential transformants were selected as indicated above, and *pbp1*genes were amplified and sequenced to confirm transformation. Minimal inhibitory concentrations (MICs) of antibiotics were determined for B258 and all transformants using the broth microdilution method according to a previously published protocol (DeLoney et al., 1999) described in Chapter 1.

Bio-Amox labeling of PBP1

Biotinylated amoxicillin (bio-Amox) was prepared by the method of Dargis et al., (1994). Whole cells were obtained from 48 h cultures from agar medium, suspended into 0.1 M sodium phosphate buffer pH 7.4, and then adjusted for consistent protein concentrations using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, Calif.). Equal protein aliquots of approximately 100 µg were reacted with bio-Amox at concentrations of 4, 2, 1, 0.4, 0.2, 0.1, 0.05, and 0.02 µg/ml for 30 min at room temperature. Proteins were then separated using sodium dodecyl sulfate-14% polyacrylamide gel electrophoresis (SDS-14% PAGE). After electrophoresis, proteins were transferred to nitrocellulose and prepared for detection by chemiluminescence as

described previously (DeLoney et al., 1999). Density readings were taken using Odyssey software (LI-COR, Lincoln, NE).

Antibiotic Competition experiment

In antibiotic competition experiments, 26695 and 26695_T whole cells were obtained from 48 h agar cultures, re-suspended into 0.1 M sodium phosphate buffer pH 7.4, and adjusted for consistent protein concentrations (~100 µg) and incubated with Penicillin G at 0.1, 1 and 10 µg/ml for 30 min at room temperature. These samples were then incubated with 2 µg/ml of bio-Amox for 30 min at room temperature. bio-Amox-labeled proteins were detected by chemiluminescence and density readings were taken as described above. The IC_{50} for Penicillin G for PBP1 was estimated using densitometry tracing data.

Homology Modeling

Homology models of the transpeptidase region of PBP1 from *H. pylori* 26695 and B258 were created using as a template the crystal structure of PBP1A from *Streptococcus pneumoniae* (Protein Data Bank, PDB Code: 2C5W, Chain A, residues 267-650; Guex et al., PDB,). The sequence of *Streptococcus pneumoniae* PBP1A was the best match to the sequence of *H. pylori* from the deposited structures in the PDB at the time of modeling. We also examined the suitability of the structure of *Streptococcus pneumoniae* PBP1B for multiple template homology modeling; however, despite the overall similar fold, there are significant loop length differences among the three structures, rendering the structure

of *Streptococcus pneumoniae* PBP1B non-optimal for use as a template. The Swiss Model server (http://swissmodel.expasy.org; Guex et al., 1997) was used to perform homology modeling of the transpeptidase region (amino acids 262-628), using complete PBP1 protein sequence from *H. pylori* strains. The transpeptidase domain of the two PBP1 proteins is 24% identical and 38% similar to that of PBP1A from *Streptococcus pneumoniae*. The homology models were visualized using the program DeepView (Guex et al., 1997) and were inspected for van der Waals clashes, secondary structure quality, and disulfide bond correctness. Comparative analyses of hydrogen bonds, salt bridges, charge and apparent pKa values were performed for the mutated amino acids between PBP1s from 26695 and B258.

Electrostatic Calculations

Electrostatic potential calculations were performed using the Adaptive Poisson-Boltzmann Solver (APBS; Baker et al., 2001) implementation within the program VMD (Humphrey et al., 1996) and the PARSE force field (Sitkoff et al., 1994). A dielectric coefficient of 4 was used for the protein whereas a dielectric coefficient of 78.54 was used for the solvent in the absence of solvent counter ions. The temperature was set to 298.15K. The dielectric surface was defined as the protein contact surface using a sphere with probe radius of 1.4 Å corresponding to a water molecule. The grid size was 129 × 129 × 129 points and the box size was 105 Å × 127 Å × 111 Å for the coarse grid and 82 Å × 95 Å × 86 Å for the fine grid.

Results and Discussion

Characterization of *H. pylori* strain B258

The MIC for clinical strain B258 is 1 µg/ml for amoxicillin, in comparison to our reference strain 26695 which has a MIC of 0.06 µg/ml (Table 4). Strain B258 had similar MICs for ampicillin and penicillin, but 2 µg/ml for cefotaxime. Sequencing of the transpeptidase domain of *pbp1* of strain B258 revealed 13 amino acid changes compared to the reference strain 26695, as shown in Figure 7a. Transformation of 26695 with the transpeptidase region of *pbp1* from B258, creating 26695_T, resulted in an increase in its MIC for amoxicillin to 1 µg/ml, equivalent to that of B258 (Table 4), suggesting that these changes in PBP1 account for the entire resistance to amoxicillin seen in B258. Strain 26695_T also had increased resistance for the 3 other β-lactams tested (Table 4).

β-Lactam	26695 MIC (µg/ml)	B258 MIC (µg/ml)	26695_T MIC (µg/ml)
Amoxicillin	0.06	1.00	1.00
Ampicillin	0.125	1.00	2.00
Penicillin G	0.125	1.00	2.00
Cefotaxime	1.00	2.00	4.00

Table 4. MIC determinations of strains 26695, B258, and 26695_T for several β-lactam antibiotics. Representative of atleast three replicates of this experiment.

Effect of various amino acid substitutions in PBP1 on amoxicillin susceptibility

Strain 26695 was transformed with various segments of *pbp1* from B258, representing clusters of amino acid substitutions (Figure 8a), and the transformants were

selected on amoxicillin containing plates. Transformant C_2+3+4, which includes nine of the observed amino acid substitutions, had a MIC of amoxicillin of 1.0 µg/ml, accounting for the entire resistance of B258. This suggests that the 4 upstream amino acid substitutions (Asn 322 Asp, Asp 336 Glu, Glu 406 Ala, and Ser 417 Thr, shown in Figure 7a) do not play a significant role in conferring amoxicillin resistance. Consistent with this conclusion, we were unable to isolate a C_1 transformant (containing Glu 406 Ala, Ser 417 Thr) on amoxicillin containing plates; these amino acids are located on the external face of the protein, close but not within the putative binding cleft (Figure 8b,c). However, transformant C_1+2 has a MIC of 0.25 µg/ml for amoxicillin, and based on the information above, would suggest that amino acid substitutions Ser 455 Asn, Val 469 Met and Phe 473 Leu are responsible for this increased resistance to amoxicillin. Transformant C_3+4 and Transformant C_4 both have MICs of 0.25 µg/ml for amoxicillin, suggesting that the 4 amino acid changes seen in cluster 3 do not contribute to amoxicillin resistance; as seen in Figure 8b,c, cluster 3 is located at a site removed from the putative binding cleft. In a separate experiment (data not shown here), a single point mutation Ser 543 Arg was created in 26695, resulting in an MIC of 0.25 µg/ml for amoxicillin, which suggests that Met 533 Ile does not contribute to amoxicillin resistance (which was confirmed by the inability of a 26695 strain transformed with a PBP1 containing only the Met 533 Ile substitution to demonstrate any increase in amoxicillin resistance, data not shown). A transformant containing Val 469 Met and Phe 473 Leu from cluster 2 in addition to Ser 543 Arg was created in 26695 and this construct had a MIC of 1.0 µg/ml, suggesting that these 3 sites were sufficient to account for all of the

Fig. 7a

```
                                  Transpeptidase domain

                                      262
26695                                 VVDEVLKQLDQLDGLKTQGYTIKLTIDLDYQRLALESL
B258                                  ---------------------------------------

        301                                                   350
26695   RFGHQKILEKIAKEKPKTNASNEDEDNLNASMIVTDTSTGKILALVGGIDYKKSAFNRATQAKRQFGSAIKPFVY
B258    ---------------------D-------------E-------------------------------------

       376                                                                    450
26695   QIAFDNGYSTTSKIPDTARNFENGNYSKNSEQNHAWHPSNYSRKFLGLVTLQEALSHSLNLATINLSDQLGFEKI
B258    ------------------------------A----------T---------------------------------

        451                                           500
26695   YQSLSDMGFKNLPKDLSIVLGSFAISPIDAAEKYSLFSNYGTMLKPMLIESITNQQNDVKTFTPMETKKITSKEQ
B258     ----N-------------M---L-----------------------------D---NI-V--------------

        526                                                                    600
26695   AFLTLSVLMDAVENGTGSLARIKGLEIAGKTGTSNNNIDAWFIGFTPTLQSVIWFGRDDNTPISKGATGGVVSAP
B258    --------I--------R-------------------------------------------------------

       601                                           650
26695   VYSYFMRNILAIEPSLKRKFDVPKGLRKEIVDKIPYYSTPNSITPTPKRTDDSEERLLF
B258    ------------------------------------------------------------
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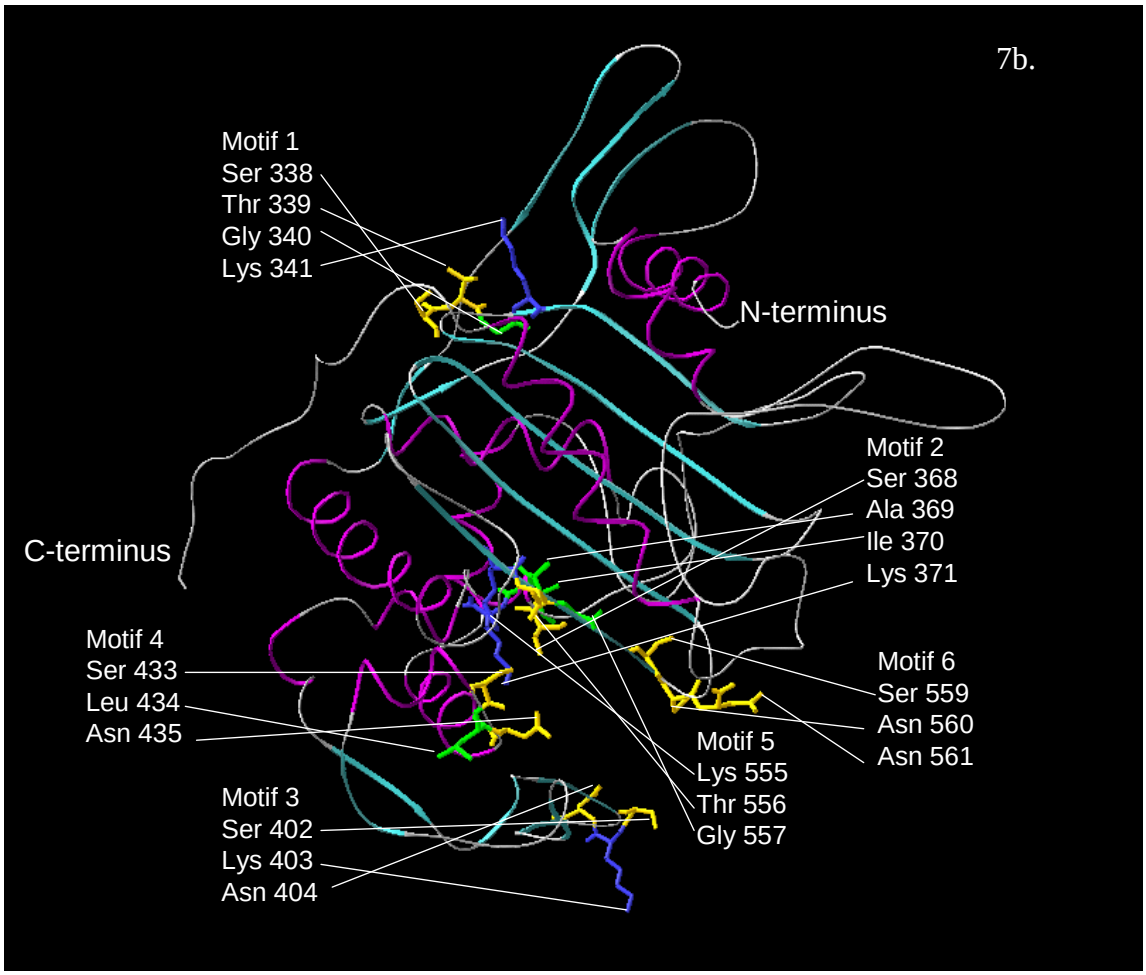


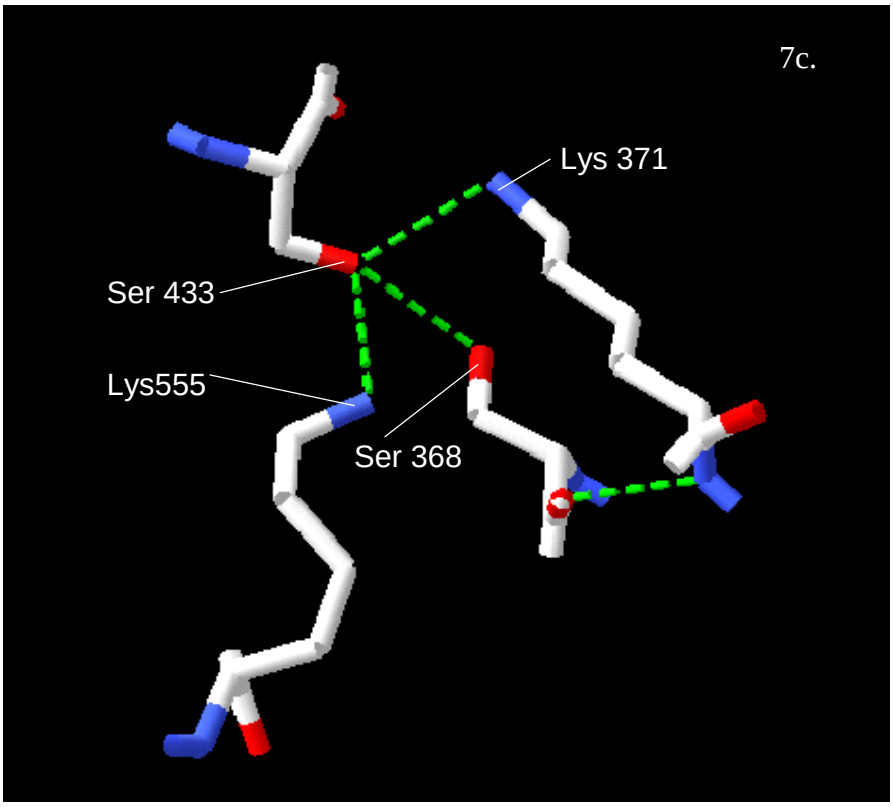


Fig. 7a. Alignment of the PBP1 transpeptidase domains from 26695 and B258. The dashed lower region indicates no change in B258 at that locus compared to 26695. Specific amino acid substitutions are denoted by the letter of the changed amino acid. Boxed amino acids indicate putative penicillin binding motifs (PBMs). Fig. 7b. Secondary structure of PBP1 transpeptidase region from 26695 depicted in ribbon representation (purple, alpha-helices; cyan, beta-sheets; gray, loops). The putative PBMs (boxed amino acids in Fig. 7a) are also shown, with amino acid side chains depicted in stick representation and colored by type (green, hydrophobic; yellow, polar; blue, polar positively charged). All other side chains have been deleted for clarity. Fig.7c. Triple hydrogen bond of putative catalytic Ser 368 with Ser 433, Lys 371, and Lys 555 within the putative binding cleft of the PBP1 transpeptidase region from 26695. Green dashed lines denote hydrogen bonds.

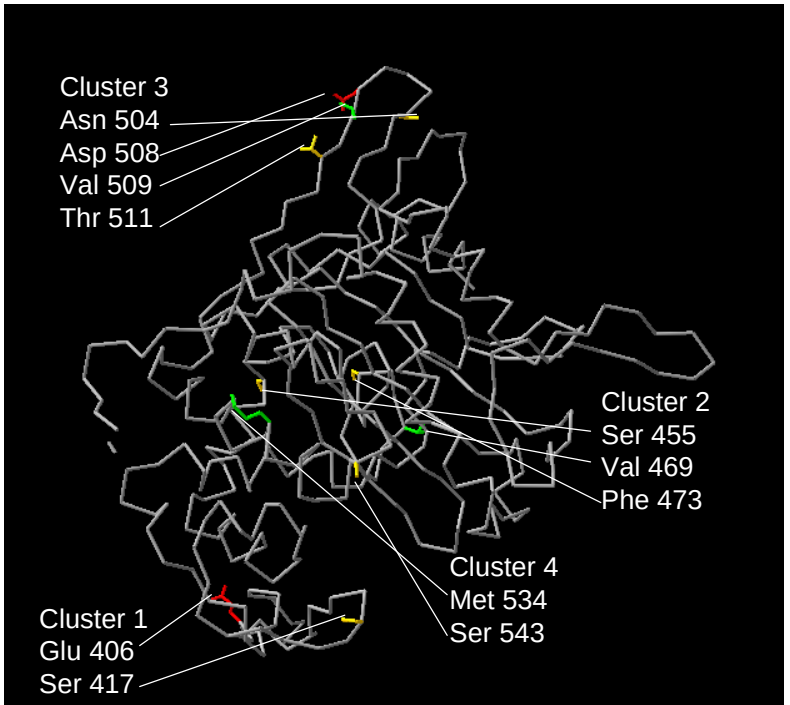
amoxicillin resistance of B258. These amino acids are located in the vicinity of the large putative binding cleft (Figure 8b,c). Several efforts to select a Phe 473 Leu + Ser 543 Arg double mutant, without Val 469 Met, have been unsuccessful.

PBP1 binding

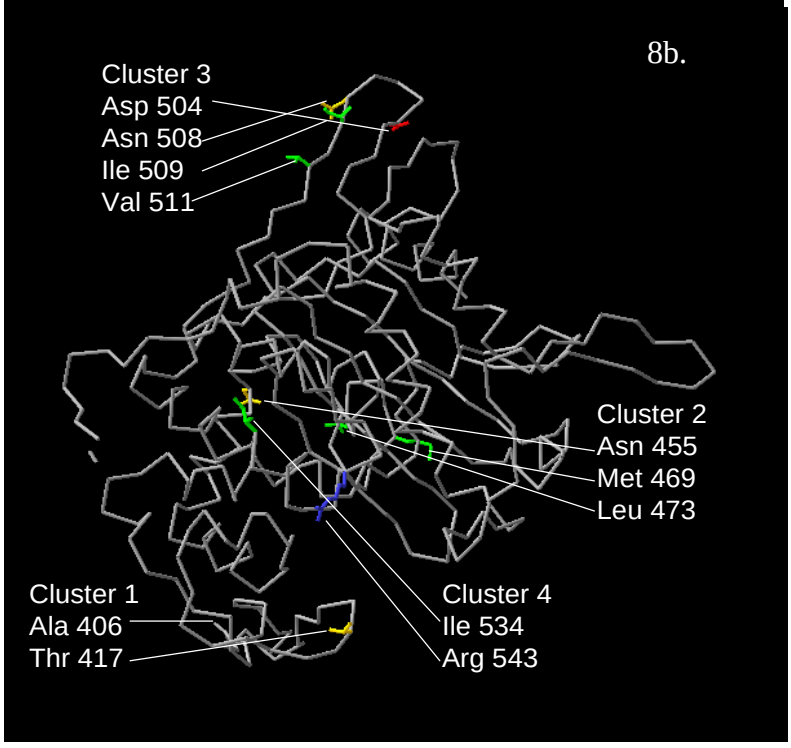
Whole cells from strains 26695, B258 and 26695_T were prepared and incubated with bio-Amox as described in the Materials and Methods section. Results of a representative experiment are shown in Fig. 9a and the data from repeat experiments analyzed quantitatively are presented in Fig. 9b. Labeling of 26695_T and B258 were identical at each bio-Amox concentration and was lower than that of 26695 for all bio-Amox concentrations <0.5 µg/ml. We also examined the affinity of the PBP1s from 26695 and 26695_T for amoxicillin using an antibiotic competition experiment. Each strain was pre-incubated with penicillin G at 0.1, 1 or 10 µg/ml for 30 min prior to incubation with 2 µg/ml of bio-Amox. The IC_{50} for Penicillin G for PBP1 in 26695 was estimated to be 0.57 µg/ml whereas it was 29.7 µg/ml for PBP1 in 26695_T.

Homology Modeling of PBP1 from *H. pylori*

Protein modeling of the transpeptidase region placed 5 out of the 6 putative penicillin-binding motifs (PBMs) (Harris et al., 2000) within a putative binding cleft (Figure 7b). The first motif however mapped away from the cleft. The Ser 543 Arg transformation results in an increase in protein net charge from +5 to +6. This charge increase results in a significant increase in the spatial distribution of positive electrostatic potential, which forms an electrostatic hotspot surrounding the putative binding cleft (Fig. 10a and 10b). The arginine at 543, although about 19 Å away from the putative catalytic Ser 368, is located in a loop in the vicinity of the large putative binding cleft and


Cluster 3
Asn 504
Asp 508
Val 509
Thr 511
Cluster 2
Ser 455
Val 469
Phe 473
Cluster 4
Met 534
Ser 543
Cluster 1
Glu 406
Ser 417

8b.


Cluster 3
Asp 504
Asn 508
Ile 509
Val 511
Cluster 2
Asn 455
Met 469
Leu 473
Cluster 4
Ile 534
Arg 543
Cluster 1
Ala 406
Thr 417

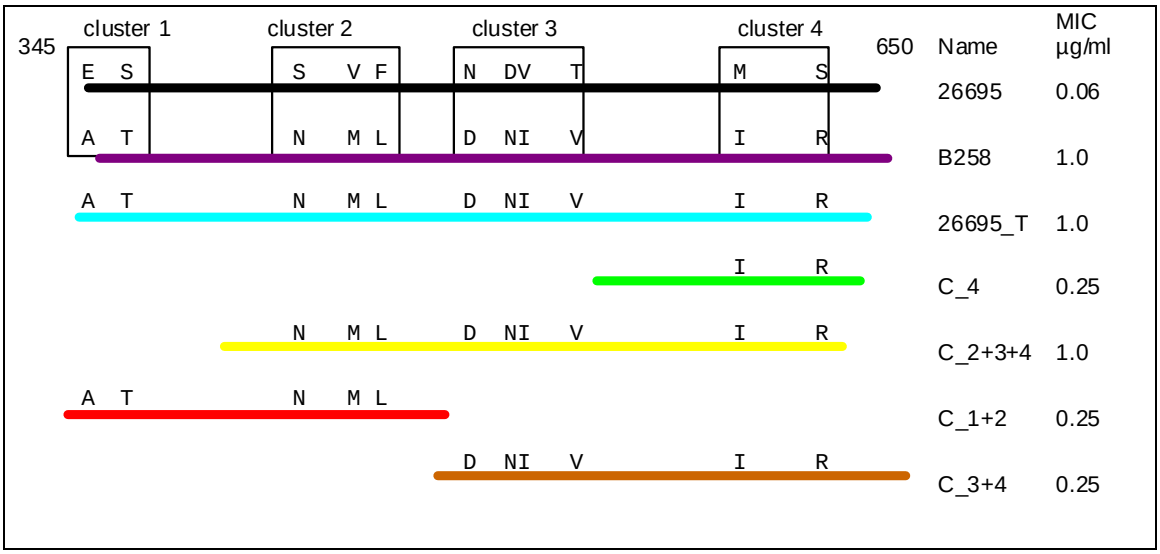
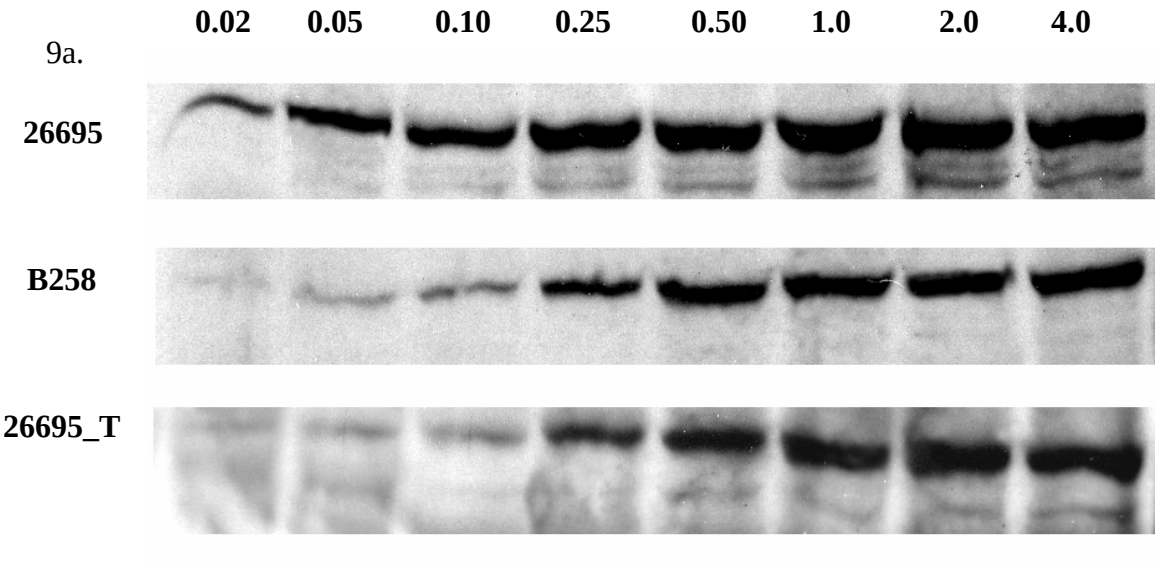


Fig. 8a. Alpha-carbon trace representation of transpeptidase region of PBP1 of 26695 with the side chains of amino acids changed in stick representation (all other amino acids are deleted for clarity). The color code is by amino acid type (green, hydrophobic; yellow, polar; blue, polar positively charged; red, polar negatively charged). Fig. 8b. Alpha-carbon trace model of transpeptidase region of PBP1 of B258 showing amino acid substitutions in a similar manner as in Fig. 8a. Fig. 8c. The amino acid substitutions seen in the PBP1 transpeptidase domain from B258 were divided into clusters based on their sequence proximity to each other. Strains 26695, B258 and 26695_T contained all clusters. Other transformants contained different combinations of clusters as indicated by the numbers in their name.

may interfere with the binding of the β-lactam antibiotic (Fig. 11). The arginine at 543 may be responsible for diverting the negatively charged β-lactam antibiotic from the site of catalysis, either through non-specific long-range electrostatic recognition or through specific short-range charge-charge interaction, or both. This type of electrostatic steering leading to recognition and binding has been discussed in the case of protein-protein interactions (Cheung et al., 2010; Wu et al., 2006; Zhang et al., 2006; Zhang et al., 2007).

The remaining substitutions in the PBP1 of B258 that had an effect on MIC, that is Val 469 Met and Phe 473 Leu, are about 20 Å away from the catalytic Ser 368, but

they are located in a loop enclosing the putative binding cleft. Our homology modeling (or the crystallographic structure of the template for that matter) does not depict protein dynamics which are more pronounced in solvent-exposed (> 20% solvent accessibility) flexible loop regions. Substitutions Val 469 Met and Phe 473 Leu belong to the loop 459-476. Met 469 and Leu 473 seen in PBP1 of 26695 have solvent accessibilities of 43% and 11%, respectively, in the modeled loop conformation (out of many possible conformations due to inherent loop flexibility). The substituted amino acids, Val 469 and Phe 473 in the case of PBP1 of 26695 have solvent accessibilities of 30% and 17%. The substitution Ser 543 Arg belongs to the loop 540-552. Arg 543 has solvent accessibility 40%. The substituted Ser 543 in the case of PBP1 of 26695 has solvent accessibility of 25%. (Solvent accessibilities were calculated in the presence of the hydrogen atoms in the model structures.) Higher solvent accessibility values correspond to amino acids in highly flexible portions of the loops. The flexible loop on which Ser 543 is located is likely to play an important role in the dynamics of protein-ligand binding. Therefore, the significant change of Ser 543 Arg, which modifies both the steric bulk as well as the charge, of this loop may conceivably affect the binding kinetics, thermodynamics, or both. The Phe 473 Leu change may result in a loss of a very weak π-cation interaction between the aromatic ring of Phe 473 and Lys 464. The steric hindrance argument also applies to substitution Ser 543 Arg, which occurs in a solvent-exposed mobile loop, in addition to the electrostatic arguments described above. Most of the other amino acid substitutions in the PBP1 of B258 were not predicted to make any significant change to the putative binding cleft. The putative catalytic serine at position 368 (Contreras-Martel



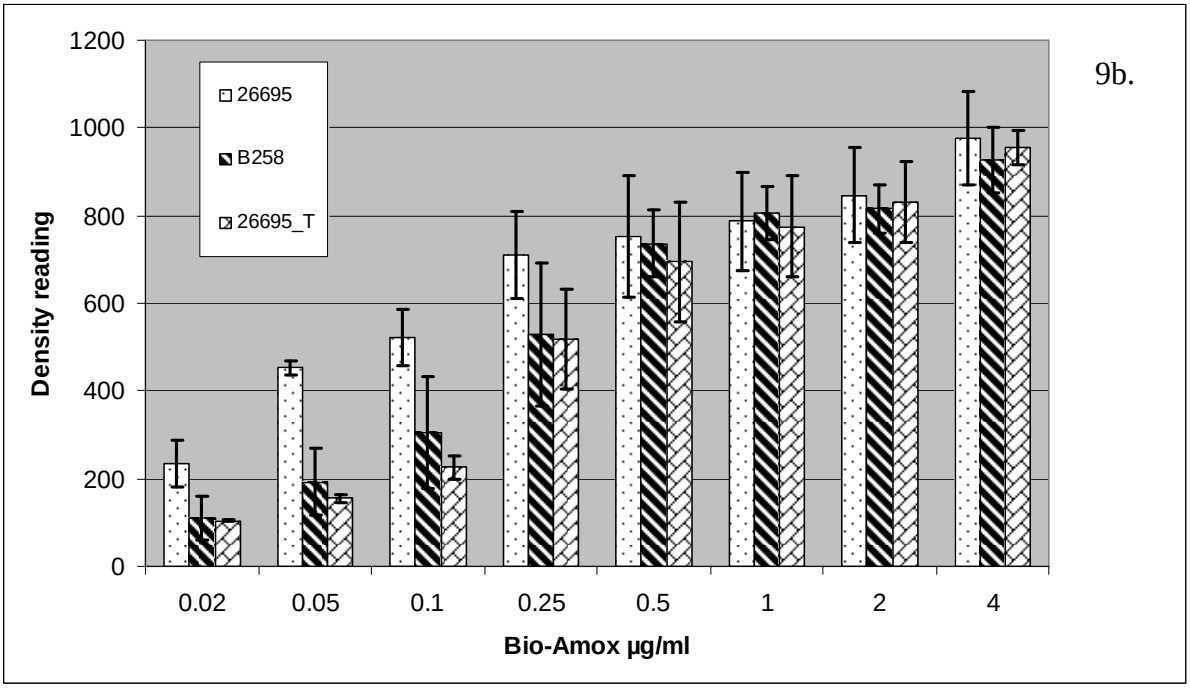


Fig. 9a. PBP1 bio-Amox binding profiles in *H. pylori* 26695, B258 and 26695_T. Numbers above the lanes indicate the amount of bio-Amox used in the experiment, in µg/ml. Fig. 9b. Densitometric comparisons of PBP1 from the 26695, B258 and 26695_T strains labeled with various concentrations of bio-Amox (shown in µg/ml on bottom axis). Bars represent the mean $\pm$ SD based on 3 separate experiments.

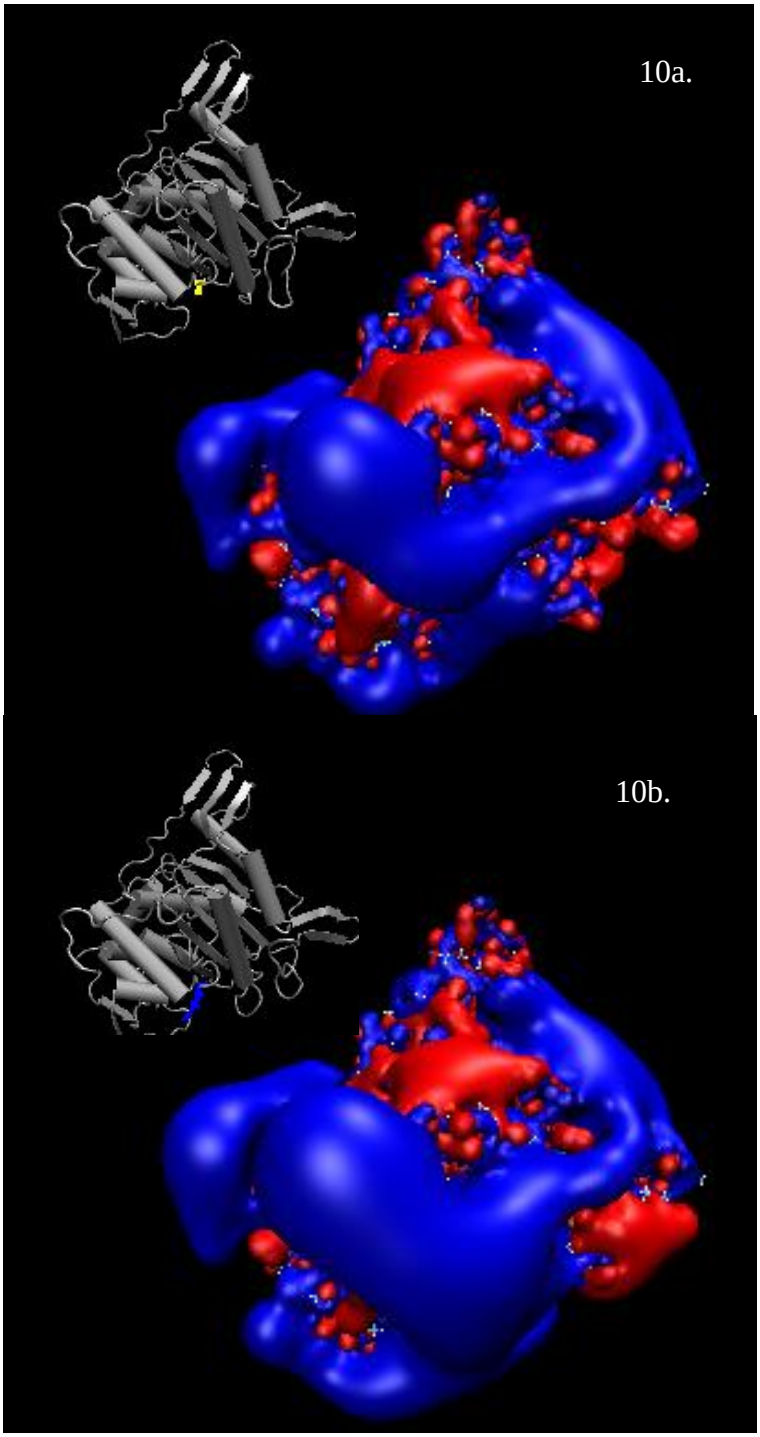


Fig. 10. Isopotential contour surfaces depicting the spatial distributions of electrostatic potentials of the transpeptidase region of PBP1. Blue and red represent positive and negative electrostatic potentials, respectively, at $\pm 2k_BT/e$ units. 10a. Transpeptidase region of PBP1 from 26695. Inset shows Ser 543 in yellow. 10b Transpeptidase region of PBP1 from B258. Inset shows Arg 543 in blue.

et al., 2006) is in the middle of the cleft (Fig. 7b) and participates in a complex hydrogen bond network (Fig. 7c). Although the catalytic Ser 368 is well-anchored by hydrogen bonds, the mobility of the surrounding loops of the catalytic cleft may be responsible for controlling ligand entry and binding, depending on the type of loop mutations. Molecular dynamics simulations are planned to study the effects of loop flexibility and mobility on the formation of the catalytic cleft.

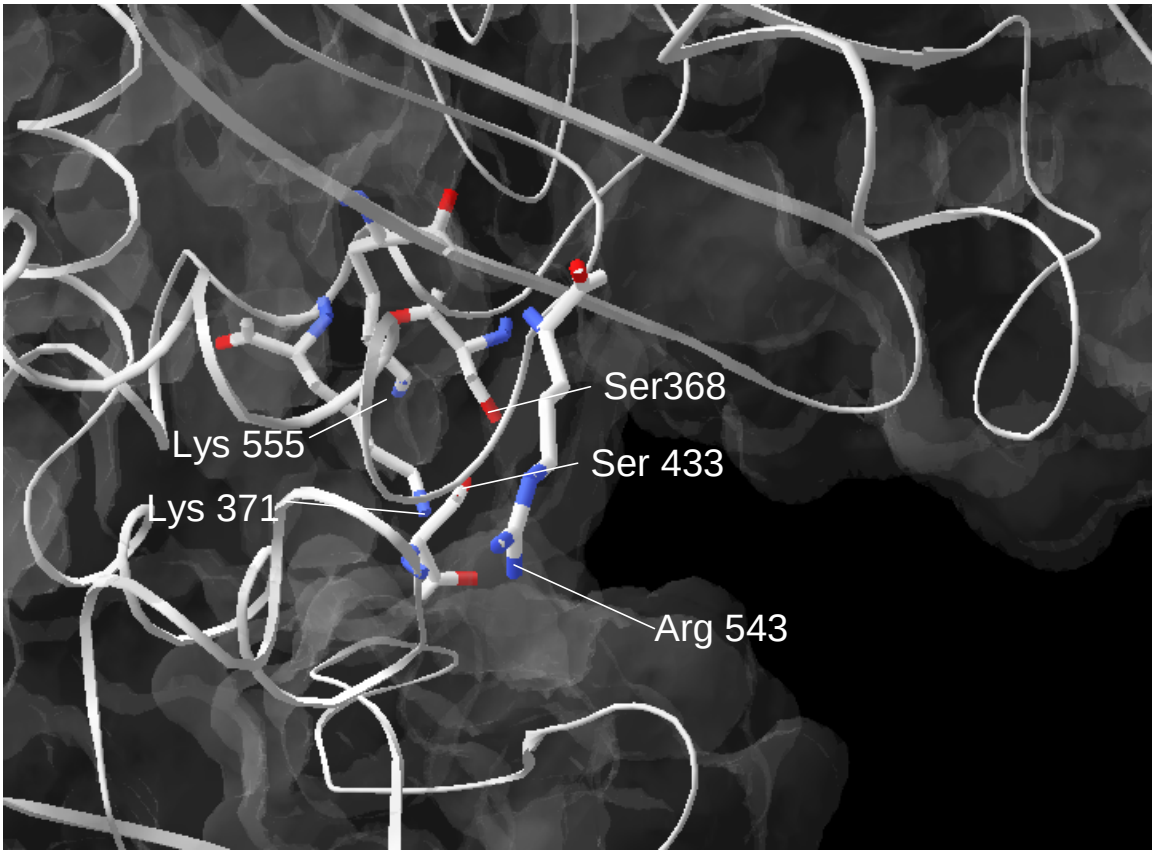


Fig. 11. Surface representation of the cleft showing amino acids within 4 Å of the putative catalytic Serine (Ser 368) of the PBP1 transpeptidase region from 26695. In addition, although Arg 543 from the superimposed structure B258 is located 18.5 Å away from the putative catalytic Ser 368, it potentially interferes with ligand entry.

Previously published data identified several mutations in PBP1 which confer resistance to *H. pylori* (Co et al., 2006; Gerrits et al., 2002 and 2006; Kwon et al., 2003; Matteo et al., 2008; Okamoto et al., 2002; Paul et al., 2001; Rimbara et al., 2007; Tseng et al., 2009) and we chose to visualize the putative effect of four of the most commonly reported mutations, namely Ser 414 Arg, Thr 438 Met, Thr 556 Tyr and Asn 562 Tyr. The mutation Ser 414 Arg results in an increased net positive charge comparable to that seen with Ser 543 Arg. The mutations of these two serines to arginines perhaps share the same mechanism of interaction with the β-lactam antibiotic, although Ser 414 Arg is located right at the entrance of the binding cleft and may be more prone to specific charge-charge interaction with the β-lactam antibiotic (Figure 12a,b). Two mutations (Thr 438 Met and Asn 562 Tyr) result in substitution with an amino acid of a significantly larger size, likely causing steric hindrance within the putative binding cleft (Figure 6a,b). Thr 556 Ser mutation is present in close proximity to putative catalytic Ser 368 and Lys 555 and likely interferes with function of Ser 368. Other amino acid substitutions did not contribute to resistance regardless of their proximity to the PBMs or the putative binding cleft (e.g., Glu 406 Ala or Ser 417 Thr).

Our initial studies demonstrated that the MICs of *H. pylori* clinical isolate B258 to ampicillin, amoxicillin and penicillin G were significantly increased (8-fold or greater) when compared to the amoxicillin sensitive reference strain 26695. On the other hand, the MIC to cefotaxime was only modestly increased. PCR amplification and sequencing of the *pbp1* gene from B258 identified 13 amino acid substitutions when compared to the *pbp1*gene from 26695. Replacement of the transpeptidase domain (amino acids 600 to

1850) of the *pbp1* gene in 26695 with the *pbp1* gene from B258 resulted in the transformant 26695_T with an MIC for amoxicillin identical to that of B258. This experiment demonstrated that the amoxicillin resistance of strain B258 is due entirely to amino acid changes in PBP1. It should be noted that besides PBP1 changes, there have been a few reports describing changes in outer membrane permeability which have affected amoxicillin sensitivity (Co et al., 2006; Kwon et al., 2003) whereas in other reports, only part of the amoxicillin resistance has been attributed to PBP1 changes and the other resistance mechanisms remain uncharacterized (Gerrits et al., 2002; Gerrits et al., 2006; Kwon et al., 2003; Okamoto et al., 2002; Paul et al., 2001; Rimbara et al., 2007). Finally, although less important than PBP1, Rimbara et al., 2008 reported that amino acid mutations in PBP2 and PBP3, in concert with amino acid changes in PBP1, increase amoxicillin resistance.

In our studies using bio-Amox labeling, transformation of 26695 with the transpeptidase domain of PBP1 from B258 demonstrates that the increased amoxicillin resistance of B258 is due to decreased binding of amoxicillin to the transpeptidase domain of PBP1. Since penicillin G, ampicillin and amoxicillin are structurally similar, it is not surprising that the altered PBP1 in B258 would bind less effectively to each of these antibiotics, resulting in increased MIC for each of these antibiotics. Using antibiotic competition experiments, we calculated the IC_{50} for penicillin to be more than 50 times higher for the 26695_T PBP1 than for the amoxicillin sensitive strain 26695. While other studies have reported that amino acid substitutions in PBP1 affect

amoxicillin resistance, this study is one of the few to demonstrate both reduced amoxicillin binding as well as calculate the IC_{50} value for the altered PBP1.

Using chimeric PBP1 transformants, we were able to eliminate or minimize the importance of most of the amino acid substitutions in PBP1 in strain B258 in conferring amoxicillin resistance, suggesting 2 (or 3) amino acid sites as critical. Although the percent identity between the homology model and its template was relatively low (24% identity, 38% similarity), the experimental data from chimeric transformants supported our results. We confirmed the importance of the Ser 543 Arg mutation using site-directed mutagenesis of the *pbp1* gene in 26695, resulting in a 4-fold increase in amoxicillin resistance. This same site has also been reported to be important for amoxicillin resistance by Kwon et al., 2003.

As seen in the projected model, penicillin binding motifs 3 and 6 are located at the entrance points of the putative binding cleft, perhaps as gate keepers for antibiotic entry (Fig. 7b). Lys 371 (motif 2), Ser 433 (motif 4) and Lys 555 (motif 5) are projected to form hydrogen bonding interactions with the putative catalytic Ser 368 (Fig. 7c). The substitution of Ser 543 with Arg 543 increases the overall charge by one unit and renders a stronger positive electrostatic field to the protein, which may interfere with the recognition and binding of the negatively charged β-lactam antibiotic (Fig. 10). The increase and change in the directionality of the positive electrostatic field caused by the Ser 543 Arg mutation may increase the recognition ability for β-lactam and divert its entry path away from the catalytic site. Another, or additional, mechanism involves

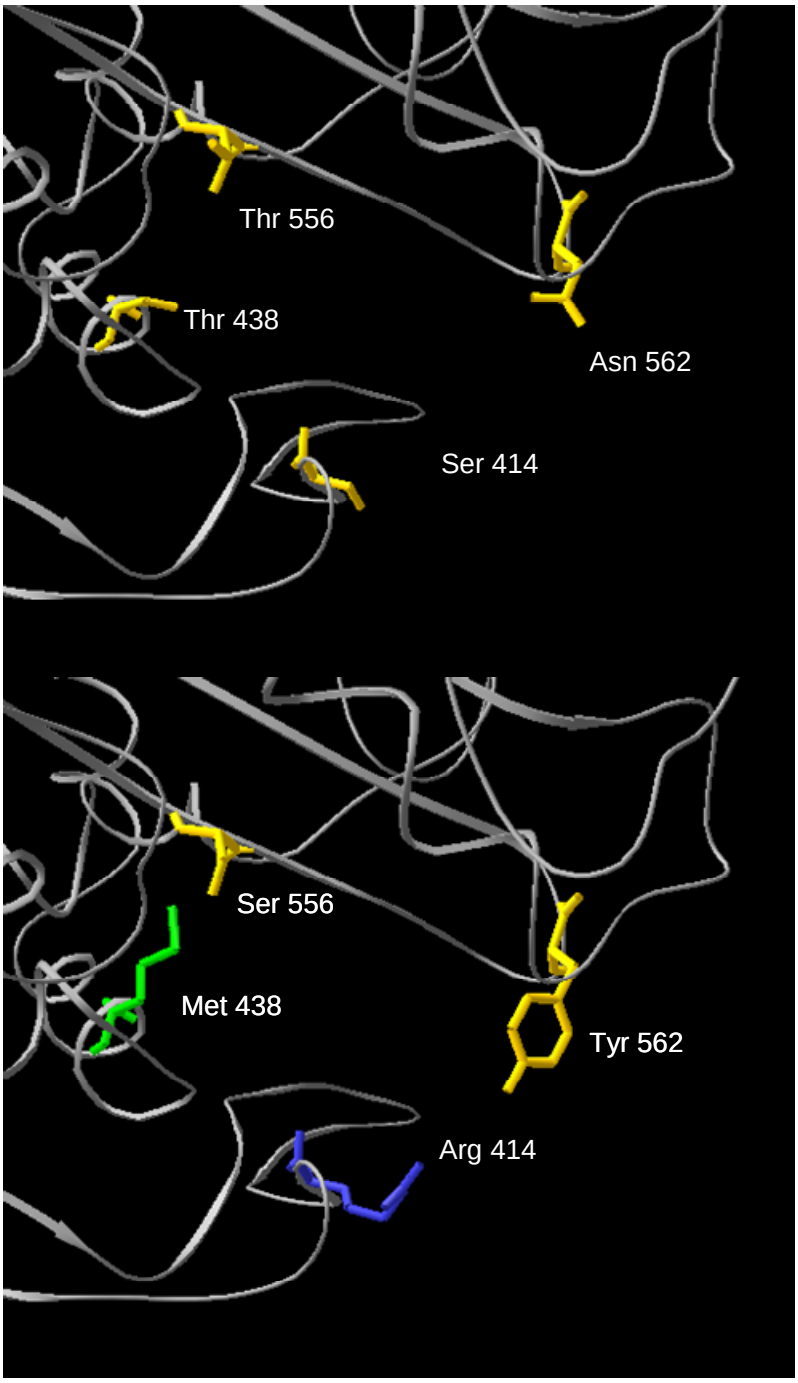


Fig. 12a. Localization and stick structure representation of 4 key amino acids within the putative binding cleft in strain 26695 (amoxicillin-sensitive). Fig. 12b. The predicted stick structure representation of unique amino acid substitutions for these 4 amino acids previously reported to increase amoxicillin resistance in various clinical strains.

possible local Coulombic attraction between Arg 543^{+} and COO^{-} of β-lactam, which may cause inaccessibility to the catalytic site.

On the other hand, the finding of a mutation at the Phe 473 Leu site is novel and may be significant because this change results in a loss of a very weak π-cation interaction which previously occurred between Phe 473 and Lys 464. Interestingly, we were unable to isolate a Phe 473 Leu and Ser 543 Arg double mutant without a corresponding Val 469 Met amino acid substitution. It may be that Val 469 Met is a compensatory mutation needed to stabilize the PBP1 protein with the Phe 473 Leu mutation.

Our data suggest that mobility of solvent-exposed loops surrounding the putative binding cleft may control ligand entry towards the catalytic Ser 368, which is anchored by a triple hydrogen bond in the base of the cleft (Figure 7b,c). Certain mutations in these loops that produce antibiotic resistance may mediate steric and electrostatic effects which hinder antibiotic access to the catalytic Ser 368. Such "active site breathing" has been recently proposed as being a possible mechanism for β-lactam resistance in pneumococci (Contreras-Martel et al., 2009).

A careful review of the amino acid changes in PBP1 in amoxicillin resistant strains in previously published studies have led to our hypothesis that only a handful of amino acid substitutions can alter amoxicillin binding - Ser 414 Arg (Gerrits et al., 2002; Gerrits et al., 2006; Paul et al., 2001; Rimbara et al., 2007), Thr 438 Met (Co et al., 2006), Phe 473 Leu (this paper), Ser 543 Arg (Kwon et al., 2003 and this paper), Thr 556 Tyr (Gerrits et al., 2006; Kwon et al., 2003; Matteo et al., 2008; Okamoto et al., 2002;

Tseng et al., 2009), and Asn 562 Tyr (Gerrits et al., 2006; Kwon et al., 2003; Okamoto et al., 2002; Rimbara et al., 2007). These amino acid substitutions have appeared as single changes with corresponding increased amoxicillin resistance (Co et al., 2006; Gerrits et al., 2002; Matteo et al., 2008) or as parts of clusters of changes (Gerrits et al., 2006; Kwon et al., 2003; Okamoto et al., 2002; Paul et al., 2001, Rimbara et al., 2007, Tseng et al., 2009 and this paper). The homology modeling demonstrates how these individual changes affect the putative binding cleft, apparently impeding amoxicillin docking. It is also clear that combinations of these specific substitutions, as seen in this paper, can additively increase the level of amoxicillin resistance found. However, the myriad of other amino acid substitutions sometimes found in PBP1 from amoxicillin resistant strains are unlikely to add to the effect seen by these specific amino acid changes.

These studies reveal that it is not the proximity of amino acid changes to PBM's per se that determines whether a substitution will confer resistance; for example, the Glu 406 Ala substitution is very close to the SKN PBM but does not affect amoxicillin sensitivity. Rather it is the impact of the specific amino acid change within the putative binding cleft which appears to be more important. Our studies also suggest that changes which have a significant effect on the structure of the protein, particularly around the putative binding cleft, can have an effect on amoxicillin binding regardless of their lack of proximity to the PBM's (for example, Ser 414 Arg and Phe 473 Leu). On the other hand, Thr 556 Ser and Asn 562 Tyr are in, or adjacent to, a PBM respectively and clearly affect amoxicillin sensitivity.

Amoxicillin resistance in *H. pylori* is increasing in the United States. A better understanding of its resistance mechanisms will allow changes in treatment strategies to prevent the occurrence of treatment failures. Furthermore, rapid screening of resistant isolates could be performed on clinical isolates using molecular techniques if the absolute number of amino acid mutations conferring resistance could be determined. In this study we suggest that currently only 6 sites seem critical, and of these 6, only three substitutions - Ser 414 Arg, Thr 556 Ser and Asn 562 Tyr - have been reported in multiple clinical isolates, suggesting these are the most common amino acid changes in PBP1 connected to amoxicillin resistance.

Chapter Five

Characterization of Resistance Mechanisms and Evolution of an In Vitro Selected Amoxicillin-Resistant *Helicobacter pylori* Strain

Introduction

Previous studies have demonstrated the role of PBP1 in low to moderate β-lactam resistance in *H. pylori* but additional resistance mechanisms must be present to achieve high levels of resistance. The evolution of the changes resulting in high level resistance has also not been established. The order in which each of these changes occur can be an important tool to determine the likelihood of specific mutations in isolates with different MICs.

To characterize all the potential mechanisms of β-lactam resistance in *H. pylori*, we isolated an amoxicillin-resistant strain with high level of resistance by continuously passing an amoxicillin-sensitive (26695) strain with an MIC of 0.06 µg/ml in increasing amounts of amoxicillin over a period of nine months. Isolates were also collected and frozen at each intermediate level of amoxicillin resistance. Initial investigations of the most resistant isolate (IS6) revealed changes in Penicillin Binding Protein 1 (PBP1), PBP2 and several porin proteins. Further analysis also indicated a decrease in uptake of penicillin in IS6 when compared to the original parental amoxicillin sensitive strain. It has been previously speculated that outer membrane proteins (OMPs) such as porins may be responsible for this decreased antibiotic accumulation (DeLoney et al., 2000).

To identify novel genes that might contribute to an increase in amoxicillin resistance, we proceeded with whole genome sequencing to identify any changes not previously identified. The genome of IS6 was used to make a short read library, which was subsequently sequenced and aligned with the database reference genome of 26695. Analyses were performed to locate mutations within the amoxicillin resistant isolate as

compared to the amoxicillin sensitive strain 26695. The role of each of these mutations in amoxicillin resistance will be systematically investigated.

Materials and Methods

Isolation of Amoxicillin resistant strains

Strain 26695, an amoxicillin sensitive strain with a fully sequenced genome (Tomb et al., 1997), was used as the starting culture to create the amoxicillin resistant isolates. Serial two-fold dilutions of amoxicillin were prepared in Brucella Broth supplemented with 10% heat-inactivated fetal bovine serum and 1% IsoVitalex in a 96 well plate. Starting with 150 µl of 16 µg/ml amoxicillin in the first well, amoxicillin was diluted to 8, 4, 2, 1, .5, .25, .125, .06, .03, .0125 and .006 µg/ml in the subsequent wells. *H. pylori* 26695, with an inoculum OD600 (optical density at 600nm) of 0.5±.05 was inoculated at a 1:100 dilution (1.5µl in 150µl, ~ 10^6 cells). The 96 well plates were grown at 37°C, with 10% CO_2 and 100% humidity. Plates were allowed to incubate for 2 days and wells were examined for visible signs of growth. The well with the highest concentration of amoxicillin showing growth was used to inoculate the next plate prepared the same as above. Isolates were saved at various time points starting with bacteria expressing an MIC of 0.50 µg/ml (MICs were determined as described previously). The process was repeated every 48-72 hours for approximately nine months and resulted in 6 isolates labeled IS1-IS6.

Sequencing of potential target genes

Previously known potential target genes of amoxicillin resistance (such as PBP 1-3, Hop A-E, etc.) were PCR amplified from the genomic DNA of IS6. Primers for amplification of the potential targets used in this study were created using the published sequence of 26695 and Vector NTI and synthesized by Sigma-Aldrich. Polymerase chain reaction was performed in an automated thermal cycler PTC100, using the Choice Taq Blue Polymerase. PCR products were run on 1% agarose gel and subsequently cut and purified using Minelute agarose purification system. Sequencing of purified DNA product was performed by Genomics core institute at the University of California Riverside.

Whole genome sequencing

Illumina genomic DNA sample prep kit (Illumina) was used to create the genomic library. All subsequent reactions were performed according to instructions. Five µg of genomic DNA from IS6 was sheared by sonication to obtain fragments of about 400bp. The ends were repaired by converting overhangs from fragmentation into blunt ends, using T4 DNA polymerase and *E. coli* DNA polymerase I Klenow. Further, an A base was added to the 3' end of the blunt phosphorylated DNA fragments using the polymerase activity of Klenow. Specific DNA adapters were ligated to the ends of the DNA fragments, to prepare them to be hybridized to a flow cell. The ligation product was purified using PCR purification kit (Promega). This purified product was submitted to the

Genome center at UCR where it was sequenced using the Illumina Genome Analyzer using single read at 32bp per read.

The data was then put through the flowcell pipeline in the form of short reads. These short reads were then assembled and analyzed for Single Nucleotide Polymorphisms (SNPs) and single base pair insertions or deletions (indels) by the Bioinformatics department at UCR.

Identifying potential targets

SNPs and single insertions and deletions were analyzed for mutations resulting in amino acid changes within genes. Mutations in the last 6 amino acids of a protein or not within a coding or promoter region were excluded. The resulting 25 potential genes were identified. PCR primers were created for each of the potential targets and synthesized by Sigma Genosys. These genes were then PCR amplified and sequenced as mentioned above.

Evolution of Mutations

Minimum inhibitory concentrations (MIC) of the six Isolates IS1-IS6 were determined as described in Chapter 2. Ten potential targets identified in the above procedure were then PCR amplified and sequenced as mentioned above in all isolates IS1-IS6. The sequencing data were analyzed to confirm the presence or absence of the target mutations in each isolate.

Transformation of PBP1

Mutant *pbp1* was PCR amplified, and purified from IS6 using the PCR protocol mentioned above. The product was run on 1% agarose gel with 0.5µg/ml ethidium bromide and isolated using Minelute gel extraction kit. The product was quantified using Beckman DU 530 UV/VI spectrophotometer and 100-150 ng was used to transform 26695 by the procedure of Wang et al. (1993) . The transformants were plated on Brucella agar plates containing 5% defibrinated sheep blood. After 48 hours the bacteria were transferred to Brucella-blood Agar plates with amoxicillin with concentrations of 0.125, 0.25 or 0.5 µg/ml. All resistant colonies were isolated and their genomic DNA purified using DNeasy tissue kit. PBP1 was PCR amplified and sequenced to confirm the presence of expected mutation.

[^{14}C] penicillin G accumulation experiments

[^{14}C] penicillin G accumulation experiments were performed using Log-phase Brucella-blood agar cultures suspended in PBS buffer to a final concentration of 3-5 x10^9 bacteria/ml and incubated with [^{14}C] penicillin G to a final concentration of 1µCi/ml at 37ºC. Half ml aliquots were removed at 1, 5, 10 and 30 mins, the bacteria were pelleted by centrifugation and washed 2 times with PBS to remove unaccumulated antibiotic. Cells were subsequently resuspended in 0.5 ml of PBS and added to 10 ml of scintillation fluid (Fisher Scientific). The amount of antibiotic accumulated was measured in counts per minute (CPM) using a Beckman LS6500 liquid scintilliation counter (Beckman Coulter)

Results and Discussion

Sequential passage of *H. pylori* 26695 amoxicillin sensitive strain in increasing levels of amoxicillin resulted in a gradual increase of over 90x in resistance. There were two reasons for using this strain; the 26695 genome has been completely sequenced (Tomb et al., 1997), making comparisons and identification easier, and this strain is amenable to transformation, whereas some other strains have proven to be difficult to transform in our lab (unpublished data). The amoxicillin sensitive strain had an MIC value of .06 µg/ml prior to the selection process, which increased to 5.5 µg/ml. The in vitro selected strain maintained this MIC value after regrowth from a freezer stock, suggesting a stable increase in MIC as compared to a transient or unstable increase. A stable increase in MIC suggests that the changes have occurred at the sequence level as opposed to changes in transcriptional levels with response to stimuli. In the process of in vitro selection various isolates were obtained starting at the 0.50 µg/ml MIC IS1 to 5.5 µg/ml MIC IS6 (Table 5). To determine precise MICs we diluted amoxicillin to increments of 0.5µg/ml starting at 1.5µg/ml to 7.5µg/ml (Table 5).

To determine the sequence changes conferring resistance to IS6, commonly known potential target genes such as PBP 1-3, Hop A-E, Mre ABC and a few other regulatory genes were sequenced and compared against the 26695 genomic sequence. After sequencing of several different genes only three genes PBP1, PBP2 and Hop C from IS6 showed any changes when compared to 26695. The sequence of PBP1 (HP0597) from IS6 showed two amino acid changes P372S and T438M. Upon sequencing the *pbp1* genes in IS1-IS5, P372S substitution appeared in IS1 whereas the

Isolate	MIC value (µg/ml)
IS1	.50
IS2	1
IS3	2.5
IS4	4
IS5	4.5
IS6	5.5

Table 5. MIC values for the various Isolates IS1-IS6.
Representative of atleast three replicates of this experiment.

T438M mutation appeared in IS4 (Table 7). The *pbp1* gene from IS6 was amplified by PCR and used to transform 26695. The MIC value was raised from 0.06 µg/ml to 0.25 µg/ml. Although significant, this change in MIC is much less than that observed in IS6 which has an MIC of 5.5 µg/ml. P372S is located adjacent to the SAIK PBM which contains the putative catalytic Ser 368 (Fig 4). T438M has been previously seen in an in vitro selected strain (Co et al., 2006) and is located near the SLN PBM (Fig. 4).

Sequence comparison of PBP2 from IS6 to amoxicillin sensitive strain revealed the E536K mutation (Fig 13). Lysine is positively charged and this change would result in an overall charge increase in PBP2. Sequence comparison of Hop C revealed a single mutation resulting in an Arginine change into a Histidine (data not shown).

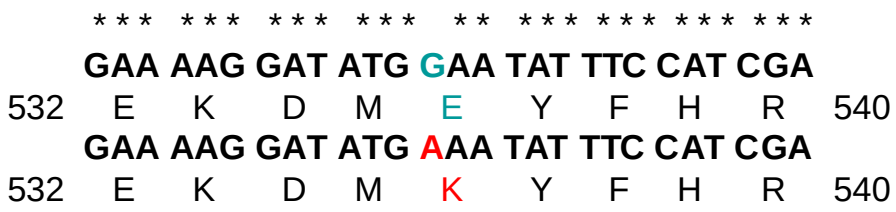


Fig. 13. Nucleic and amino acid sequences of PBP2 from *H. pylori* amoxicillin sensitive strain 26695 and IS6. The Guanine to Alanine change results in a Glutamate to a Lysine change.

IS1 has an MIC value of 0.5 µg/ml and appears to only contain P372S mutation which is in close proximity to the putative catalytic Ser 368. There are also 6 distinct MIC levels within the isolates and the changes identified so far cannot account for all of the resistance. After sequencing of several known potential targets, it became obvious that there were other changes in the genome which had not been detected. In order to locate these changes, whole genome sequencing was employed. Unlike de novo genome sequencing, a template based genome sequencing was employed as a cost efficient way to locate changes within the entire genome of IS6. This method uses short reads to cover the entire genome and then a reference genome is used to assemble these reads. *H. pylori* 26695 is NC_000915 genome 1.667 Mbps. The sequencing coverage over the entire genome was greater than 30X.

Gene	locus	Mutation	Gene description
HP0571	603952	P→S	Hypothetical Ded A uncharacterized membrane associated protien
HP0597	631507 631706	T→M P→S	Penicillin Binding protein 1
HP0602	638854	70 bp upstream	3 methyl adenine DNA glycosylase
HP0607	643852 644591	D→E L→F	Acriflavine resistance protein AcrB Multidrug efflux protein cooperates with TolC.
HP0912	966081	R→H	Omp 20 also known as Hop C
HP0946	1007964	G→W	Hypothetical Nhac; Na+/H+ antiporter; Anion permease, coserved membrane protein.
HP0958	1007441	D→T	Posttranscriptional regulator that modulates Flagellin A (Fla A)
HP1565	1647429	E→K	Penicillin Binding Protein 2
HP0181	188235	Indel. Middle of coding region	Putative colicin V protein
Hp1167	1233499	Indel. Middle of coding region	OMP 3. Putative outer membrane protein Hof H

Table 6. Mutations in the ten potential target genes for conferring resistance to *H. pylori*. sequence was used from the NCBI database. Since *H. pylori* has a comparatively small

Data mining identified approximately 30 SNP mutations and approximately 50 single and double amino acid Indels. Further analysis of SNPs to exclude mutations within the coding region that did not translate in an amino acid change and mutations in the last 6 amino acids of the coding region, eliminated about one third of the SNPs. Indels were further analyzed to exclude any changes that were in the last 6 amino acids of the coding region or not present within a coding, or promoter region of a gene, allowing us to eliminate two thirds of the indel mutations. Twenty five SNP and indel mutations remained. These mutations were then sequenced in the *H. pylori* amoxicillin sensitive

strain used to create isolates IS1-IS6. Fifteen of these mutations were present in the amoxicillin sensitive strain eliminating them as potential resistance targets.

Name	IS1	IS2	IS3	IS4	IS5	IS6
HP0571	-	-	-	-	-	**+**
HP0597 T→M	-	-	-	**+**	+	+
HP0597P→S	**+**	+	+	+	+	+
HP0602	-	**+**	+	+	+	+
HP 0607	-	**+**	+	+	+	+
HP0912	-	**+**	+	+	+	+
HP0946	-	**+**	+	+	+	+
HP0958	-	**+**	+	+	+	+
HP1565	-	-	-	-	**+**	+
HP0181	**+**	+	+	+	+	+
HP1167	-	-	-	-	**+**	+

Table 7. Order in which each mutation appeared in the different isolates. The bold + sign indicates a mutation that occurred in that isolate.

A total of ten genes remained as potential resistance targets including PBP1, PBP2 and HopC. The data on these genes is shown in Table 6. These mutations were further sequenced in IS1-IS 6 to determine the order in which they appeared (Table 7). IS1 contains the P372S mutation as well as mutation in HP0181 which is a putative colicin V protein. The combination of the two may explain the MIC of 0.5µg/ml in IS1. Of the ten mutations, five mutations appeared first in IS2. Some of these mutations may not confer amoxicillin resistance. IS3 does not show any new mutations, which might indicate that there are larger insertions or deletions present which were not identified by the data mining process. IS4 contains the T438M mutation in PBP1. IS5 presents the

PBP2 mutation and HP1167 which is a previously uncharacterized putative outer membrane protein. IS6 contains HP0571 mutation which is the uncharacterized membrane associated protein. Several of these genes are potentially good targets that have been previously uncharacterized in the context of amoxicillin resistance.

It can be challenging to use data mining to look for indels of unspecified lengths. So in order to cover the entire genome and acquire any information that data mining may have missed, we scrolled through the alignment of genome with its short bp reads using samstools on the biocluster server at University of California, Riverside. The alignment file showed a high quality of the short reads and the alignment. Scrolling through the alignment helped to identify four deletions of about 20-30 bps. Two of these mutations are not likely to contribute to amoxicillin resistance. One of these is present at the end of the transposase gene and the other is in Cag 7 protein which plays a role in pathogenesis. Two genes that showed deletions were putative outer membrane proteins; Omp 19, which contains multiple mutations, and Omp 9, which contains a short 6 bp deletion.

The results of this study have identified several mutated outer membrane proteins potentially conferring amoxicillin resistant to the in vitro selected isolates. To test this hypothesis accumulation studies were performed using [^{14}C] penicillin G. These experiments shows an ~30% decrease in uptake by IS6 (Fig. 14). These results suggest that mutations in outer membrane proteins have decreased uptake of β-lactams, which would be expected to significantly effect the MIC.

Further studies are being done to characterize the effect of individual potential target genes on conferring resistance to β-lactams. This study identifies at the genome

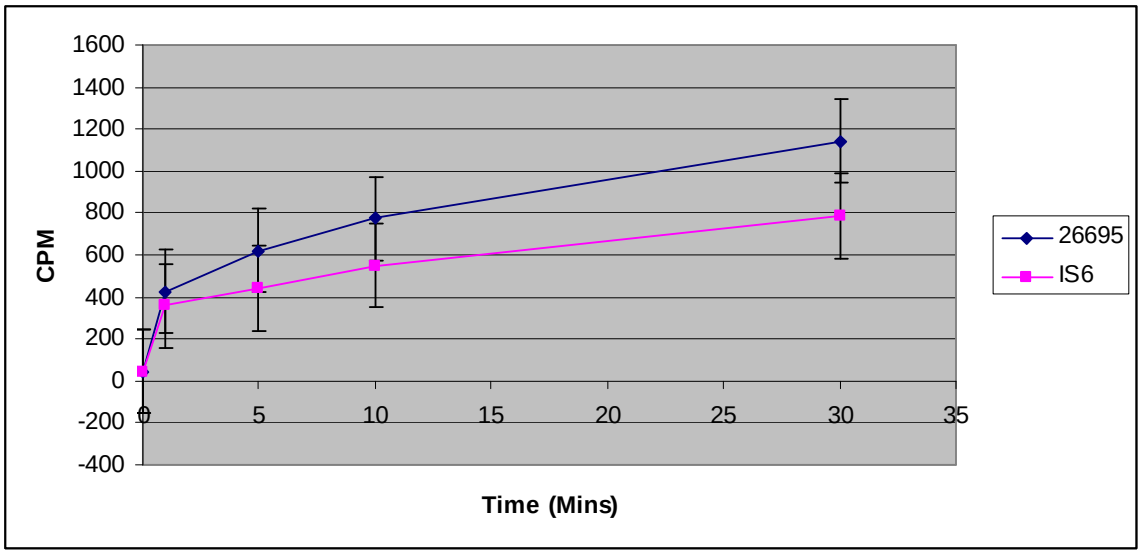


Fig 14. Accumulated [14C] penicillin G profiles of 26695 and IS6. Data bars shown are averages of two separate experiments plus range.

level the type of changes that can occur when *H. pylori* is subjected to amoxicillin over a long period of time. Some of these changes are consistent with previously shown data, while others are potentially new targets which when mutated could confer resistance to β-lactams. It is interesting to note that four out of the 12 potential targets are outer membrane proteins. Another gene of interest is HP0607 (AcrB) which cooperates with TolC; a TolC pump conferring resistance to metronidazole has also been shown to work on β-lactams (Amsterdam et al., 2005). Homologs of penicillin binding protein 2 have been shown to confer resistance to β-lactams in other bacteria (Jonge et al., 1992); however it has not been shown to contribute significantly to β-lactam resistance in *H. pylori*.

Previous studies on clinical isolates, except for B258, have only been able to explain part of the amoxicillin resistance seen in *H. pylori*. However looking at all the

changes that have occurred in the genome of IS6 should help to account for all genes contributing toward resistance. The genome sequencing approach would be more efficient in an in vitro selected strain since it makes the number of changes manageable; however with the right analysis tools it could potentially be used to identify changes in clinical strains as well.

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