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Evolution of Amoxicillin Resistance of *Helicobacter pylori* *in vitro*: Characterization of Resistance Mechanisms

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ABSTRACT

Objectives: *Helicobacter pylori* is the major cause of peptic ulcers and gastric cancer in humans. Treatment involves a drug cocktail, typically including amoxicillin. Increasing levels of resistance to amoxicillin contribute to treatment failures. Higher levels of resistance are believed to be due to multiple genetic mutations, likely due to higher fitness cost of resistance mutations in a single gene. The progression of β -lactam resistance mutations in multiple genes and the effects of those mutations at various intervals has not been investigated. In this study we examined the evolution of amoxicillin resistance in *H. pylori* when exposed to increasing concentrations of amoxicillin *in-vitro*.

Methods: Using prolonged incremental exposure to amoxicillin, we isolated 5 strains each of which had progressively higher levels of resistance. Using a whole genome sequencing approach, we identified the genome wide changes which occurred in the most resistant isolate relative to the parental amoxicillin sensitive strain. By sequencing these changes in each isolate, we were able to map the order in which these mutations emerged. Minimum inhibitory concentrations (MIC) were determined for each isolate, and amoxicillin uptake and efflux studies were used to evaluate possible contributions to amoxicillin resistance. To further characterize the contributions of the identified mutations, strains containing individual mutations were created through allelic exchange and evaluated using MIC and efflux studies.

Results: Whole genome sequencing revealed a total of 11 mutations within 5 isolates. The individual sequencing of each isolate showed a gradual accumulation of mutations at

each interval correlating with the increment seen in the MIC values of each isolate. While some of these mutations were found in genes previously connected with amoxicillin resistance in *H. pylori* (penicillin binding proteins 1 and 2, and outer membrane protein HopC), other mutations were identified in outer membrane proteins and efflux proteins not previously associated with amoxicillin resistance in *H. pylori*. These novel mutations were supported by decreased accumulation of ¹⁴C-penicillin within the cell and increased sensitivity to amoxicillin in the presence of efflux pump inhibitors.

Conclusions: Our results provide evidence for the complexity of the evolution of amoxicillin resistance in *H. pylori*, and identify a variety of genes which contribute to this resistance. The data also indicate that certain families of genes might be more susceptible to amoxicillin resistance mutations than others.

Introduction

Colonization of the stomach by *Helicobacter pylori* is often asymptomatic; however, this colonization can trigger an inflammatory response responsible for a majority of cases of peptic ulcers, stomach cancers and B-cell mucosa-associated lymphoid tissue (MALT) lymphomas.^{1,2} Two or more antibiotics are generally used to treat persistent and symptomatic infections of *H. pylori* but ultimately may not be effective due to increasing rates of antibiotic resistance.³ *H. pylori* has been shown to possess a high mutation rate⁴ with specific point mutations in the bacterial genome resulting in increasing levels of resistance to various antibiotics⁵, including the β -lactams⁶. Moreover, recent studies have shown that unsuccessful eradication of *H. pylori* after treatment with amoxicillin leads to an increase of amoxicillin resistance in these bacteria.⁷

Previous studies have demonstrated that certain point mutations in penicillin-binding protein-1 (PBP1) confer resistance to β -lactam antibiotics in *H. pylori*.⁸⁻¹² However, these studies, as well as others demonstrate that PBP1 point mutations can only account for low levels of amoxicillin resistance, suggesting that high levels of resistance must require additional resistance mechanisms.^{13,14}

In this study we track the emergence of a high level amoxicillin resistant strain of *H. pylori* ~~*in vitro*~~ *in vitro*. Using whole genome sequencing and comparison to the parental amoxicillin sensitive strain, we identified changes that occurred within the bacterial chromosome affecting amoxicillin susceptibility. By sequencing strains with intermediate levels of amoxicillin resistance which emerged on the path to the highly resistant strain, we established the sequence in which these mutations originated. Our results provide evidence for the complexity of the evolution of amoxicillin resistance in *H. pylori*, and identify a variety of genes which can contribute to this resistance.

Materials and Methods

Bacterial strains and culture conditions

H. pylori ATCC strain 26695 was used as the amoxicillin-sensitive reference strain and the parental strain to create the ~~*in vitro*~~ *in vitro* resistant isolates. All bacterial strains were incubated at 37°C in a humidified 10% CO₂ incubator. Cultures were streaked for isolation on Brucella agar (Becton-Dickinson Microbiology, Cockeysville, MD) supplemented with 5% defibrinated sheep blood (Colorado Serum Company, Denver, CO). 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 were prepared by re-suspending 48 h bacterial 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 genes from 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™ DNA polymerase (Denville Scientific, Metuchen, NJ). PCR products were run on 1% agarose gels (Biorad, Hercules, CA) and subsequently cut and purified using the Minelute agarose purification system (Qiagen Inc., Valencia, CA). Sequencing of purified DNA products was performed by the Genomics Core facility at the University of California, Riverside. Sequence analysis and alignments were performed using Vector NTI.

Isolation of amoxicillin resistant strains

Strain 26695, an amoxicillin sensitive strain with a fully sequenced genome¹⁵, was used as the starting culture to create amoxicillin resistant isolates. Serial two-fold dilutions of amoxicillin were prepared in Brucella broth supplemented with 10% heat-inactivated fetal bovine serum and 1% IsoVitalax in a 96 well plate. Wells contained sequentially-diluted amoxicillin concentrations ranging from 16 mg/L to 0.006 mg/L. *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, ~ 10⁶ cells). The 96 well plates were incubated at 37°C in a humidified 10% CO₂ incubator for 2 days and wells were examined for visible signs of growth. A 1.5 µl aliquot of bacteria from the well with the highest concentration of amoxicillin showing growth was used to inoculate the next plate prepared the same as above. The process was repeated every 48-72 h for approximately nine months. Isolates were saved at various time points and colony purified starting with bacteria expressing a minimal inhibitory concentration (MIC) of 0.50 mg/L of amoxicillin and resulted in 5 isolates labeled IS1-IS5. MICs to amoxicillin of all isolates were determined using the broth microdilution method according to a previously published protocol.¹⁶ MIC values were also determined in 0.5 mg/L increments for amoxicillin concentrations between 1 and 8 mg/L.

Whole genome sequencing

An Illumina genomic DNA sample prep kit (Illumina, Inc., San Diego, CA) was used to create the genomic library. All subsequent reactions were performed according to instructions. Five µg of genomic DNA from the final isolate 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 a PCR purification kit (Promega). This purified product was submitted to the Genome center at UCR where it was sequenced with the Illumina Genome Analyzer using single read at 32bp per read. The data were then put through the flowcell pipeline in the form of short reads. These short reads were assembled and analyzed for Single Nucleotide Polymorphisms (SNPs) and single base pair Insertions or Deletions (InDels) by the Bioinformatics department at UCR. The alignment put forth was also visually inspected to locate any changes missed by data mining.

Identifying potential targets and their evolution

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. Potential target genes were sequenced in the parental strain 26695 to identify mutations present prior to the *in-vitro* amoxicillin resistant isolation process. PCR primers were created for each of the potential targets and synthesized by Sigma Genosys. The potential target genes were then PCR amplified from the genomic DNA of each isolate and sequenced as mentioned above. The sequencing data were analyzed to confirm the presence or absence of the target mutations in each isolate.

Transformation of *Helicobacter pylori*

Transformation of 26695 was achieved by electroporation, following a previously established protocol by Wang et al.⁵. Competent cells were prepared by growing 26695 on Brucella agar supplemented with 5% defibrinated sheep blood for 24-36 h. Cells were isolated and washed three times with 20% glycerol, pelleting for 5 min at 3100 x g between washes. Prepared cells were then combined with ~500ng purified PCR amplified DNA, transferred to a 1mm gap electroporation cuvette and incubated on ice for 10 minutes. Using an ECM 630 electroporator, cells were transformed at 2.5kV, 600 Ω , and 25uF, which resulted in a time constant ~13msec. Cells were re-suspended in 200uL SOC media and plated onto Brucella-sheep blood agar, and recovered for 36-48 h. Cells were scraped from the plate and re-suspended in sterile PBS followed by plating on Brucella-sheep blood agar containing either 0.125 μ g/ml or 0.25 μ g/ml amoxicillin. Individual colonies were isolated and targeted genes were sequenced to verify mutation.

[¹⁴C] penicillin G accumulation experiments

[¹⁴C] penicillin G accumulation experiments were performed using log-phase Brucella-blood agar cultures of 26695, IS3 and IS5 suspended in phosphate-buffered saline, pH 7.4 (PBS) buffer to a final concentration of 3 - 5 x 10⁹ bacteria/ml, and incubated with [¹⁴C] penicillin G to a final concentration of 1 μ Ci/ml at 37°C. Half ml aliquots were removed at 1, 5, 10 and 30 min, the bacteria were pelleted by centrifugation and washed 2 times with PBS to remove un-accumulated antibiotic. Cells were subsequently re-suspended in 0.1 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 scintillation counter (Beckman Coulter).

[¹⁴C] penicillin G efflux experiments

~~For the assay using starved cells, 4 ml bacterial cultures of IS5 were suspended in PBS buffer to a final concentration of 3-5 x10⁹ bacteria/ml, and incubated for 2 h at 37°C. The cell suspension was then divided into two halves, centrifuged, and one half was re-suspended in PBS and the other in Brucella broth containing 10% heat-inactivated fetal-bovine serum and 1% IsoVitaleX. The cells were incubated with [¹⁴C] penicillin G at a final concentration of 1 μ Ci/ml at 37°C, and 400 μ l aliquots were removed at 1, 5, 10, 20-~~

~~and 30 min. The cells were then washed and analyzed for [¹⁴C]penicillin G accumulation as mentioned above.~~

~~For the assay using~~ Using actively growing cells, 4 ml bacterial cultures of isolate IS5 were suspended in PBS buffer to a final concentration of 3-5 x 10⁹ bacteria/ml. The cell suspension was divided into two halves and one half was incubated with 40 μM CCCP (carbonyl cyanide m-chlorophenylhydrazone) for 15 min before the addition of [¹⁴C] penicillin G to a final concentration of 1μCi/ml at 37°C to both halves. Half ml aliquots were removed at 1, 5, 10 and 30 min. The cells were then washed and analyzed as mentioned above.

Efflux pump inhibitor assay

Using the microdilution MIC method mentioned above, susceptibility of 26695, IS3, IS4 and IS5 to amoxicillin was studied in the presence or absence of NMP (Naphthylmethyl-piperazine) or PAβN (phenyl-arginine-b-naphthylamide) at 100 mg/L. For IS3, IS4, and IS5, MIC values between 1-8 mg/L were determined in increments of 0.5 mg/L. For transformants containing individual mutations, MIC values between 0.03-16 mg/L were determined in 2-fold dilution increments.

Results:

Amoxicillin resistant isolates and mutations in each isolate. Sequential passage of *H. pylori* 26695 in increasing levels of amoxicillin resulted in a gradual increase in resistance culminating in a strain (Isolate 5, IS5) which expressed an amoxicillin MIC 64x higher than the parental strain's MIC value of 0.06 mg/L (see Figure 1). In the process of ~~*in vitro*~~ *in vitro* selection, various isolates were obtained and colony purified starting with isolate IS1 with an MIC of 0.50 mg/L and ending with IS5 with an MIC of 4.0 mg/L (Figure 1).

Since the 26695 *H. pylori* genome has been completely sequenced¹⁵, this strain was an ideal candidate for our studies since it would make comparisons and identification of

potential amoxicillin resistance genes easier to identify. Each of the *in-vitro* *in vitro* selected amoxicillin isolates (IS1 – IS5) maintained their MIC value after re-growth from freezer stocks, suggesting the presence of stable mutations, rather than transient or unstable changes which would be difficult to characterize. A stable increase in MIC suggested that the changes occurred at the genomic level as opposed to changes in transcriptional levels with response to stimuli.

Initial studies focused on the IS5 isolate. To determine the sequence changes conferring resistance to IS5, potential target genes such as PBPs 1-3, Hop A-E, and Mre ABC genes were sequenced and compared to those in the parental strain 26695. However, only three of these genes, *pbp1*, *pbp2* and *hopC*, from IS5 showed amino acid changes when compared to 26695 (see Table 1). The sequence of PBP1 (HP0597) from IS5 showed two amino acid changes: P372S and T438M. Upon sequencing the *pbp1* genes in the 5 isolates, IS1-IS5, the P372S substitution appeared in IS1 whereas the T438M mutation appeared first in IS4 (Table 1). Sequence comparisons of PBP2 in each of the 5 isolates (IS1-IS5) and parental strain 26695 revealed the E536K mutation only in IS5 (Table 1). Sequence comparisons of Hop C in each isolate and 26695 revealed a single mutation resulting in the conversion of an arginine to a histidine, R302H (Table 1), which appeared first in IS2 (Table 1).

Judging by the level of total resistance, and the presence of 5 isolates with distinct MICs, it seemed plausible that there were other changes which had not yet been detected. In an attempt to locate those changes, whole genome sequencing of IS5 was employed. Unlike *de novo* genome sequencing, a cost-efficient template-based short read genome sequencing was employed. Short reads are assembled against a previously published reference genome of *H. pylori* 26695 NC_000915 which is 1.667 Mbps. The sequence coverage over the entire genome was greater than 30X.

Data mining identified approximately 30 SNP mutations and approximately 50 single and double amino acid Insertions or Deletions (InDels). Further analysis of SNPs excluded mutations within the coding region that did not translate into amino acid changes, and

mutations in the last 6 amino acids of the coding region; this analysis 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 four SNP and InDel mutations remained. These mutations were then sequenced in the *H. pylori* amoxicillin sensitive parental strain 26695, to ensure that the changes observed were not present before selection of resistant isolates. Fifteen of these mutations were present in the amoxicillin sensitive parental strain eliminating them as potential resistance targets.

It can be challenging 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 basepair reads using samtools 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. These were also present in the parental strain 26695 and were not further analyzed.

A total of nine genes and eleven mutations emerged as potential resistance targets including PBP1, PBP2 and HopC. The loci of these changes, the precise amino acid change(s) that occurred, and the function of these genes are shown in Table 1. Two genes contain two mutations each, namely PBP1 and acriflavin resistance protein (AcrB). Further sequencing of these mutations in IS1-IS5 showed the order in which they evolved (Table 1). IS1 contains the P372S mutation in PBP1 as well as a mutation in HP0181 which is a putative colicin V protein. Of the eleven mutations, four mutations, AcrB, Hop C, Hypothetical Na⁺/H⁺ antiporter and Flagellin A, appeared first in IS2. IS3 has an additional mutation in AcrB and a mutation in the promoter region of DNA glycosylase. IS4 contains the T438M mutation in PBP1, while IS5 contains mutations in PBP2 and HofH, a putative outer membrane protein.

[¹⁴C] penicillin G accumulation

In order to determine whether amoxicillin resistance could be attributed to changes in antibiotic access to its periplasmic target, we performed radiolabelled penicillin G accumulation experiments. We had determined in previous studies^{16,17} that penicillin G could be used as a surrogate β -lactam drug for amoxicillin because it had similar binding affinities for PBP1. As shown in Figure 2, at each time point examined, isolates IS3 and IS5 accumulated less penicillin than the parental amoxicillin sensitive strain 26695. Over the 4 time points examined, penicillin G accumulation levels in IS3 averaged 82.5% of that seen in 26695, while IS5 had accumulation levels averaging 68% of that of 26695.

Changes in penicillin accumulation could result from either reduced entry of antibiotic into the periplasm because of altered porin proteins (such as HopC), or the active efflux of antibiotic from the periplasm due to an efflux pump mechanism. To address these possibilities, the [¹⁴C] penicillin G accumulation assay was performed on isolate IS5 in the presence or absence of the proton pump inhibitor CCCP as described in the materials and methods section. However, *we did not detect any difference in penicillin accumulation for isolate IS5 with or without CCCP treatment (data not shown).* *H. pylori* IS5 did not grow in the presence of CCCP. Alternatively,

~~In a separate experiment, a culture of IS5 was “starved” by incubation for 2 h at 37°C in PBS. Following starvation, the cell suspension was split in half, centrifuged, and half the cells were re-suspended in Brucella broth with serum and IsoVitaleX and the other half were re-suspended in PBS; each half was then examined using the [¹⁴C] penicillin G accumulation assay. The results (Figure 3) show that the initial uptake of antibiotic was the same for both cultures; however, a gradual increase in penicillin G accumulation was observed in the cells suspended in PBS whereas the cells suspended in growth media showed a lower accumulation of penicillin G (approximately 25% less than that seen in starved cells).~~

~~Finally, we examined~~ the effect of efflux pump inhibitors (EFIs) on amoxicillin susceptibility was examined using isolated strains ~~s~~ and the broth microdilution MIC method described previously. To increase sensitivity in this assay, 0.5 mg/L increments were used between 1-8 mg/L amoxicillin. ~~Initially, the~~ The MICs of amoxicillin for strains 26695, IS3, IS4 and IS5 were determined when incubated in the presence or absence of either 100 mg/L of NMP or PA β N. As shown in Table 2, the MIC for strain 26695 was unaffected by the presence or absence of EFIs. In contrast, isolate IS3, which contains two mutations in AcrB, was more sensitive to amoxicillin when grown in either EFI, reducing the MIC by 1.0 mg/L (from 2.5 to 1.5). Similarly, isolates IS4 and IS5 were also slightly more amoxicillin sensitive in the presence of an EFI, with an MIC change of 1.0 mg/L for both IS4 (3.5 to 2.5) and IS5 (4.0 to 3.0).

Transformation and mutagenesis of 26695

Based on the studies with isolated strains IS1 – IS5, we were particularly interested in assessing the effect of individual proteins with mutations on amoxicillin sensitivity, particularly PBP1, PBP2, HopC, HofH and AcrB. We have previously reported that PBP1 and ~~shown that~~ HopC mutations affect amoxicillin sensitivity^{13,18}; In this study, ~~w~~ We were able to ~~create~~ individual transformants containing the mutated PBP1, HofH and AcrB mutations found in IS5, but were unable to select a PBP2 mutant transformant. As shown in Table 3, These individual transformant strains containing mutated PBP1, HofH or AcrBs each increased amoxicillin resistance 4x over that of the parental strain 26695. ~~In addition~~ Although, the presence of the efflux pump inhibitor ~~either EFI, NMP, or PA β N,~~ did not have a significant impact on the resistance provided by either PBP1 or HofH. ~~However,~~ a dramatic reversion to the 26695 levels of susceptibility was noticed for AcrB mutants when grown in the presence of ~~either~~ EFINMP.

Discussion

The goals of this study were to characterize the evolution of amino acid changes in *H. pylori* genes when exposed to increasing levels of amoxicillin *in vitro*, and to identify the mechanisms of resistance contributed by these mutated genes. As described in the results section we successfully isolated 5 amoxicillin resistant strains, with progressively increasing resistance levels and identified a total of 11 amino acid changes within 9 genes which we believe account for the majority of the observed amoxicillin resistance. In addition, using individual mutants, this study has demonstrated the significance of specific mutations within PBP1, HofH and AcrB that impact amoxicillin susceptibility.

IS1 has an MIC value of 0.5 mg/L and contains two mutations. One mutation is P372S in HP0597 (PBP1) which is located adjacent to the penicillin binding module (PBM) sequence SAIK which contains the putative catalytic Ser 368 in the middle of the putative catalytic site.¹⁸ This mutation at this site suggests that it interferes with the binding of Ser 368 to amoxicillin. The second mutation is in HP0181 which is a Colicin V protein. This is a frame-shift mutation, a deletion of a cytosine, 86 amino acids into a 226 amino acid protein, potentially knocking out the function of the protein. It is not apparent how mutations in this protein might play a role in conferring resistance to β -lactams.

IS2 has an MIC value of 0.1 mg/L, and was found to contain 4 additional mutations in comparison to strain IS1. D131E is the first of two mutations occurring in HP0607 AcrB protein (also known as HefC), a multidrug efflux protein which cooperates with two other proteins forming a multidrug efflux pump (discussed further below). A previously known target, HP0912, also known as Omp20 or HopC contains a single point mutation at R302H. We have previously demonstrated that mutations in this gene increase resistance in *H. pylori* and decrease the level of penicillin accumulation within the cell.¹³ A point mutation is found in HP0946, a conserved membrane protein and a homolog of NhaC; Na⁺/H⁺ antiporter; anion permease. A direct connection has not been made between this protein and β -lactam resistance; however differences in expression levels of similar ion transport proteins have been seen when exposed to amoxicillin.¹⁹ The point mutation seen in HP0958, a posttranscriptional regulator for Flagellin A (FlaA) protein, seems like

an unlikely target to confer resistance to β -lactams; however a study has shown that two genes involved in production of flagella are differentially expressed when the cells are exposed to amoxicillin.¹⁹

The third isolate IS3 shows an MIC value of 2.5 mg/L and possesses two additional changes from above. HP0602, a 3 methyl DNA glycosylase, possesses a single base pair change in its promoter region. There is no evidence that this gene plays any role in conferring resistance to β -lactams. The L378F amino acid substitution is the second change in AcrB protein.

The penicillin accumulation assays showed that IS3 and IS5 had a marked decrease in the amount of antibiotic present within the cell when compared to the parental strain 26695. Accumulation assays however do not distinguish between decrease in uptake, binding or presence of an efflux mechanism. Initial attempts to identify efflux mechanisms using the proton motive force inhibitor CCCP in IS5 were unsuccessful as *H. pylori* did not grow in CCCP, but similar results were seen in other bacteria with efflux mechanisms.²⁵

~~This led us to investigate alternative methods to detect the influence of an active efflux pump. Bacterial starvation experiments showed a decrease in penicillin accumulation upon end of starvation suggesting the possible contribution of activation of efflux pumps. This is consistent with the understanding that *H. pylori* would have difficulty maintaining a proton motive force when incubated in PBS.~~²⁶

More directly, the MICs of IS3, IS4 and IS5 were decreased when exposed to either of 2 efflux pump inhibitors (EFIs), again indicating that part of the amoxicillin resistance shown might be related to an efflux mechanism. Hirata et al.²⁷ reported that the MIC for clarithromycin was decreased in *H. pylori* in the presence of PA β N, the same EFI used in this study. Since the effects of these EFIs decreased the MIC by 1.0 mg/L in IS3, and the same amounts in IS4 and IS5, we would suggest that the presence of the 2 AcrB mutations in these 3 isolates is responsible for this level of amoxicillin resistance. Previous studies have shown that a single V-F mutation in AcrB protein of *E. coli* increases its resistance to several antibiotics.²⁸ The crystal structure of AcrB protein in *E.*

coli shows a symmetric trimer of the transporter.²⁹ Using homology modeling we compared the AcrB protein in *H. pylori* to the AcrB protein in *E. coli*. The L378F mutation mapped within a loop toward the inside of the cavity, a site which might affect amoxicillin efflux through this protein. The Leu to Phe change results in a protrusion towards the center of the cavity. Considering the fact that AcrB operates as a trimer, this mutation would be presented 3 times within the complex.

Using a 26695 strain transformed with AcrB containing both mutations, [D131E](#) and [L378F](#), there was a 4x increase in amoxicillin resistance. Moreover, this resistance is abolished in the presence of ~~EFH~~, [such the efflux pump inhibitor as PAβN](#) and NMP, further supporting the hypothesis that AcrB functions as an efflux protein. Previous studies have suggested that AcrB homologs function as inner membrane efflux proteins cooperating with a periplasmic efflux protein (AcrA) and an outer membrane efflux protein (TolC). Bina et al.²⁰ identified three putative restriction-nodulation-division (RND) efflux systems in *H. pylori* – *hefABC*, *hefDEF*, and *hefGHI* – representing analogs of the AcrAB-TolC RND efflux system in *E. coli*; however, they concluded that active efflux does not affect antibiotic resistance in these bacteria. In contrast, Amsterdam et al.²¹ reported that mutations in the TolC (*hefA*) protein of *H. pylori* resulted in metronidazole resistance²¹, while Liu et al.²² reported that *hefA* is involved in multi-drug resistance in *H. pylori*. In a related strain *H. hepaticus*, *hefA* was found to increase resistance to amoxicillin.²³ Recent studies by Trainor et al.²⁴ demonstrated that the HefC efflux pump in *H. pylori* provides some resistance to bile salts and ceragenins. Collectively, this study and previous studies point to a role for active efflux pumps in *H. pylori* resistance to various compounds, including antibiotics.

IS4 contains a single additional mutation T438M in PBP1, which has been previously characterized and shown to have a 4 fold increase in resistance to β -lactams. This mutation has been shown to map within the putative binding cleft of PBP1 of *H. pylori*.¹⁸

Two additional mutations are present in the isolate IS5. One of these mutations E536K is present in HP1565 commonly known as PBP2. Because this mutation results in the

substitution with the positively charged amino acid lysine, this change would result in an overall positive charge increase in PBP2. This increased positive charge outside of the binding site can result in deflection of the antibiotic from its intended location. It has been previously shown that a charge increase in a penicillin binding protein can result in an increase in resistance.¹⁸ The region around the E536K mutation seems to be conserved in some related bacteria (data not shown), suggesting functional importance of this site within PBP2. The role of PBPs other than PBP1 in conferring resistance to β -lactams has been previously shown in several other bacteria.³⁰ A G228W mutation is found in HP1167 which is a putative outer membrane protein known as Omp3 or HofH. This point mutation occurs at a highly conserved glycine residue and would likely [affect impact protein structure and potentially function](#). The presence of this mutation transformed into strain 26695 increases amoxicillin resistance 4x. This might explain the decreased penicillin accumulation seen in IS5 relative to IS3 (Figure 2) – however further studies are needed to document this role for Omp3.

In conclusion, we hypothesize that the majority of high level amoxicillin resistance in Isolate 5 (IS5) can be explained by the combined effects of amino acid changes occurring in PBP1, PBP2, HopC, -AcrB, and Omp3. It remains possible that additional mutations, not identified [in-by](#) our sequencing strategy, can also contribute to amoxicillin resistance in IS5. [The evolution of amoxicillin resistance arising first in PBP1 is consistent with the many reports attributing low level amoxicillin resistance to amino acid changes in this protein.](#)

The pattern of high levels of β -lactam resistance in *H. pylori* and other bacteria has been shown to involve a variety of different genes from different classes.

-In *H. pylori*, cell division proteins have the highest percentage (~39 %) of essential genes, as well as cell envelope proteins (~25 %) as compared to outer membrane proteins (only ~3% are shown to be essential).³¹ PBP1 is potentially an essential protein with an upper threshold of mutations before normal cell function is affected. A mutation resulting in decreased binding of PBPs to β -lactams could decrease its functionality, limiting the extent to which amino acid changes can be made, and placing selective pressure on other

Table 1. Mutations in nine potential target genes which confer amoxicillin resistance to *H. pylori*. MIC determinations and presence of various mutations which appeared in isolates IS1 to IS5. The bold + sign indicates the mutation first occurred in that isolate.

Gene and Mutation	Locus	Gene description	26695	IS1	IS2	IS3	IS4	IS5
MIC mg/L			0.06	.5	1	2.5	3.5	4
HP0597 T438M	631507	Penicillin Binding protein 1	-	-	-	-	+	+
HP0597 P372S	631706		-	+	+	+	+	+
HP0602 70 bp upstream	638854	3 methyl adenine DNA glycosylase	-	-	-	+	+	+
HP0607 D131E	643852	Acriflavine resistance protein AcrB; Multidrug efflux protein cooperates with TolC	-	-	+	+	+	+
HP0607 L378F	644591		-	-	-	+	+	+
HP0912 R302H	966081	Omp 20 (Hop C)	-	-	+	+	+	+
HP0946 G434W	1007964	Hypothetical NhaC; Na ⁺ /H ⁺ antiporter; Anion permease, conserved membrane protein.	-	-	+	+	+	+
HP0958 D98Y	1017441	Posttranscriptional regulator that modulates Flagellin A (Fla A)	-	-	+	+	+	+
HP1565 E536K	1647429	Penicillin Binding Protein 2	-	-	-	-	-	+
HP0181 (indel)	188235	Putative colicin V protein	-	+	+	+	+	+
HP1167 G228W	1233499	OMP 3. Putative outer membrane protein Hof H	-	-	-	-	-	+

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Table 2. MIC values (mg/L) of various isolates for amoxicillin in the presence or absence of an efflux pump inhibitor (EFI) at 100 mg/L.

Strain	MIC with no EFI	MIC with PAβN	MIC with NMP
26695	.06	.06	.06
IS3	2.5	1.5	1.5
IS4	3.5	2.5	2.5
IS5	4	3	3

Table 3. MIC values (mg/L) of mutagenized 26695 strains for amoxicillin in the presence or absence of an efflux pump inhibitor (EFI) at 100 mg/L.

Strain	MIC with no EFI	MIC with NMP
26695	.06	.03
PBP1-T	.25	.125
HofH-T	.25	.25
AcrB-T	.25	.06

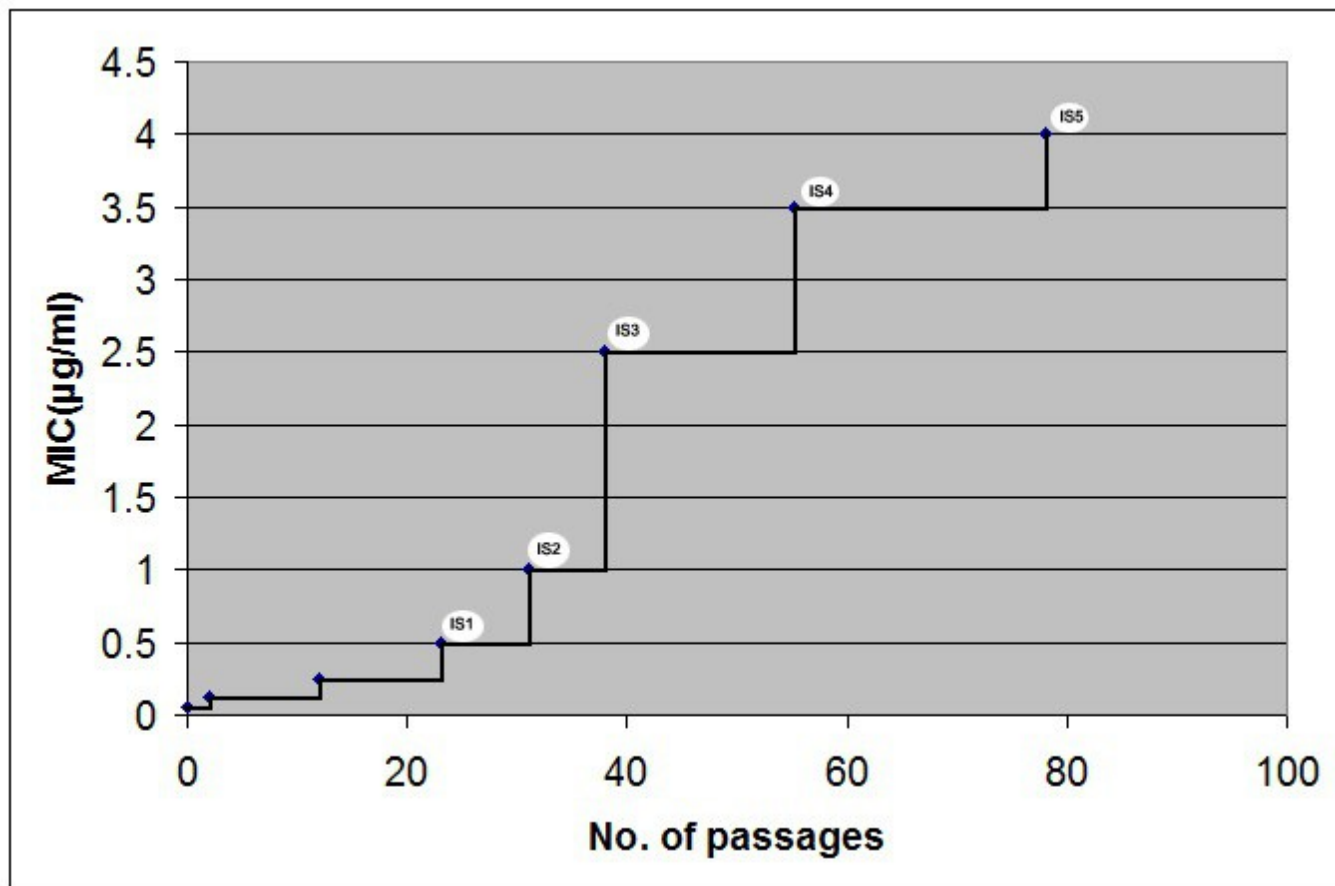


Figure 1. Evolution of amoxicillin resistance in *H. pylori* 26695 by sequential passage in increasing levels of amoxicillin. At various levels of amoxicillin resistance, bacteria were isolated and colony purified, starting with isolate IS1 with an MIC of 0.50 mg/L and ending with IS5 with an MIC of 4.0 mg/L.

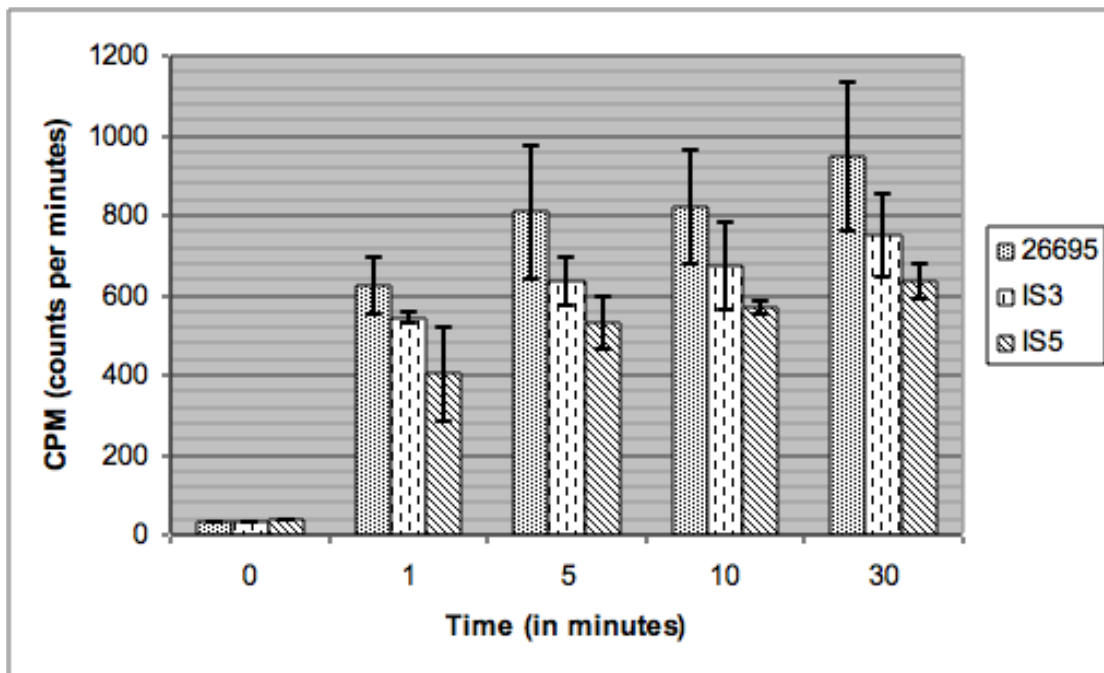


Figure 2. Isolates IS3 and IS5 accumulate less [¹⁴C] penicillin G than 26695 at each time point examined. Data shown represent the mean \pm standard deviation based on at least 3 separate experiments.

Figure 3. Influence of culture conditions on accumulation of [¹⁴C] penicillin by strain IS5. IS5 was first “starved” by incubation for 2 h in PBS; half the culture was then centrifuged and re-suspended in Brucella broth with serum and IsoVitaleX; the other half was re-suspended in PBS. The top graph shows a representative experiment; the bottom graph shows the average difference between the two culture conditions based on 3 replicate experiments using a paired t-test.

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Transparency

None to declare.

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