Role of eukaryotic Sel-1 like repeat containing genes in Helicobacter pylori evolution and pathogenesis.

Kalyani Putty
University of Louisville

Follow this and additional works at: https://ir.library.louisville.edu/etd
Part of the Biology Commons

Recommended Citation
https://doi.org/10.18297/etd/1169
ROLE OF EUKARYOTIC SEL-1 LIKE REPEAT CONTAINING GENES IN
HELICOBACTER PYLORI EVOLUTION AND PATHOGENESIS

By
Kalyani Putty, B.V.Sc & A.H.
Acharya N.G.Ranga Agricultural University, 2005

A Dissertation
Submitted to the Faculty of the Graduate School of the University of Louisville in
Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

Department of Biology, Division of Molecular, Cellular and Developmental Biology,
Program on Disease Evolution
University of Louisville
Louisville, Kentucky

December 2010
ROLE OF EUKARYOTIC SEL-1 LIKE REPEAT CONTAINING GENES IN
HELICOBACTER PYLORI EVOLUTION AND PATHOGENESIS

By
Kalyani Putty, B.V.Sc & A.H.
Acharya N.G.Ranga Agricultural University, 2005

Dissertation Approved on
November 29, 2010
By the Following Dissertation Committee

Dr. Awdhesh Kalia, Ph.D., Assoc. Professor, Dept. of Biology, University of Louisville.

Dr. Michael H. Perlin, Ph.D., Professor, Dept. of Biology, University of Louisville.

Dr. Micah Worley, Ph.D., Asst. Professor, Dept. of Biology, University of Louisville.

Dr. Douglas E. Berg, Ph.D., Alumni Professor, Dept. of Molecular Microbiology, Washington University in St. Louis.

Dr. Yousef Abu Kwaik, Ph.D., Bumgardner Chair of Molecular Pathogenesis, Dept. of Microbiology and Immunology, University of Louisville
DEDICATION

To Ajay – my light at the end of the tunnel!
ACKNOWLEDGEMENTS

Firstly, I express my heartfelt gratitude to my advisor Dr. Awdhesh Kalia for his great instructions, patience and diligent guidance. It has been a long road and his continuous support is greatly appreciated. I would like to extend a special thanks to my committee members, Drs. Douglas Berg, Yousef Abu-Kwaik, Michael Perlin and Micah Worley for their suggestions, and wonderful cooperation. I thank the present and past lab mates for lending an excellent helping hand when needed, especially Sarah Marcus for her tremendous contributions to the HcpC study. My deepest thanks to Dr. Palaniappan Sethu, and Dr. Rosendo Estrada, for help with FACS assays. Special thanks to Dr. Christopher Price (Abu-Kwaik Laboratory) and Dr. Michael Perlin for help with Real time PCR experiments. I also thank Dr. Swathi Arur, UT MD Anderson Cancer Center and Dr. Douglas Berg, Washington University in St. Louis for kindly providing several key reagents/tools for my study. A very special thanks to Dr. Micah Worley and his lab members (Josh Thornbrough and Tom Hundley) for sharing their cell culture supplies; my dear friend, Jinny Paul for encouragement, criticism and advice. I thank the Dept. of Biology for giving me the opportunity and financial support to achieve my goals. Last but not the least, none of this would have been possible without the excellent support and strength provided by my family. Very special thanks to my wonderful husband, Dr. Phani Kumar Patibandla for his unending cooperation and for giving me the strength to fulfill my dreams.
ABSTRACT

ROLE OF EUKARYOTIC SEL-1 LIKE REPEAT CONTAINING GENES IN
HELICOBACTER PYLORI EVOLUTION AND PATHOGENESIS

By
Kalyani Putty, B.V.Sc & A.H

November 29, 2010

Background: Helicobacter pylori (Hp) establishes life-long gastric infection in billions of humans, and is often responsible for diseases such as peptic ulcer and gastric cancer. Cumulative actions of genetic drift and natural selection over several millennia sculpted the present Hp population structure, which is characterized by extreme genetic diversity and striking geographic clustering of genotypes. Natural selection is more commonly imprinted in DNA sequences of Hp proteins that interact with host components; however, in most instances biological relevance of selection during Hp infection remains unknown. Here, I attempted to elucidate the consequence of natural selection in two different contexts: (1) on the preservation of duplicated genes in Hp genome; and (2) lineage-specific adaptive evolution in Hp virulence protein HepC.

Principle Findings: I characterized the molecular evolutionary dynamics of paralogs, hcpC and hcpG, which belong to the Hp Sel1-like gene family. hcpG genomic analyses identified three distinct states in natural Hp populations, whereby hcpG was either
deleted, pseudogenized or encoded highly polymorphic alleles. In contrast, full-length hcpC alleles were conserved in all genomes. Although positive selection was detected in the phylogenies of hcpG and hcpC indicating that both genes had evolved under pressure to diversify, the intensity of selection was much stronger on hcpG than hcpC. The contribution of hcpC to Hp fitness, in the AGS cell culture infection model, was significantly greater than hcpG; however, both genes together demonstrated an additive effect on Hp fitness during infection (24 hrs p.i.: \( S_{\Delta hcpC} = 0.264 \) vs. \( S_{\Delta hcpG} = 0.074 \), \( P<0.01 \); \( S_{\Delta hcpC} \) or \( S_{\Delta hcpG} \) vs. \( S_{\Delta hcpC::DhcpG} = 0.431 \), \( P<0.01 \), where \( S = \) coefficient of median fitness reduction). Furthermore, HcpC was necessary and sufficient for optimal surface expression of Heat-Shock Protein B (HspB), a major contributor to Hp virulence, specifically during infection, and functionally compensated for the lack of HcpG. In contrast, HcpG was only required for optimal HspB expression during early infection, and was unable to compensate for the lack of HcpC during later phases. Thus, a stable, genetically redundant, epistatic and overlapping yet non-reciprocal functional relationship emerged between hcpC and hcpG: natural selection favored retention of the ancestral hcpC function and sub-functionalization by fixation of loss-of-function mutations in hcpG following its origin in the Hp genome.

Earlier studies from my lab showed that the HepC protein, which also belongs to Hp Sell-like gene family, interacted specifically with human cytoskeletal protein Ezrin, and that lineage-specific positive selection changed HepC-Ezrin interaction affinity. How might alterations in HepC-Ezrin interaction affect progression of Hp infection? As a first step I established that HepC was indeed biologically relevant and contributed significantly to Hp fitness during infection (\( P=0.05 \)). Furthermore, PCR-array analyses
suggested that identical molecules of the human cytoskeletal pathway were differentially up or down-regulated by genetically diverse *Hp* isolates during infection, and that HepC likely inhibited key components of the human cytoskeletal machinery during infection. Thus HepC (possibly via its interaction with Ezrin) likely contributes a key regulatory role that might determine the pace and trajectory of *Hp* infection.

**Conclusion:** Collectively, my thesis proposes a novel mechanism through which *natural selection* favors the emergence of a stable state of genetic redundancy among duplicated genes, *hepC* and *hepG* that contribute significantly to *Hp* infection. This work also establishes a framework within which to further clarify the role of lineage-specific selection in fine-tuning *Hp*-host interactions.
## TABLE OF CONTENTS

ACKNOWLEDGEMENTS ................................................................. iv

ABSTRACT .................................................................................... v

LIST OF FIGURES .......................................................................... xi

LIST OF TABLES ........................................................................... xiii

INTRODUCTION .............................................................................. 1

I. Historical aspects of *Helicobacter pylori* .................................. 1

II. Epidemiology and clinical outcome of *H. pylori* infection .... 1

III. Genetic diversity in *H. pylori* .................................................. 2

IV. Population genetic structure of *H. pylori*: role of positive selection .... 4

V. Eukaryotic like Sell-like repeat (sir) containing gene family in *H. pylori* ................................................................. 6

VI. Expansion of *H. pylori* sir gene family by gene duplication .......... 8

VII. Host cytoskeletal dysregulation following *H. pylori* infection .... 10

SPECIFIC AIMS ............................................................................. 13

MATERIALS AND METHODS ....................................................... 15

I. *Helicobacter pylori* culture and maintenance .......................... 15

II. DNA Extraction, PCR-conditions, and DNA sequencing .......... 16
III. Computational Biological Analyses ............................................... 19
IV. Genetic Engineering of H. pylori ............................................... 23
V. Cell Culture and Antibodies ....................................................... 34
VI. Immuno blotting ......................................................................... 36
VII. Growth Kinetics and Fitness Assays ............................................. 37
VIII. Reverse-transcription PCRs ..................................................... 40
IX. Fluorescence Activated Cell Sorting Analyses ............................. 44

RESULTS ....................................................................................... 49

Evolution of Stable Genetic, and Functionally Non-Reciprocal Redundancy Driven by Positive Selection in Duplicated Sel-I like Genes of H. pylori ........................................ 49

H. pylori strain – specific srp genes ............................................... 49
Genetic rearrangement at hcpC locus is unique to H. pylori strain 26695 ...... 52
Strain specific distribution of H. pylori srp gene, hcpG ............................. 53
Unique DNA sequence polymorphisms and pseudogenization of hcpG ............................................................. 53
Variations in the number and distribution of Sel-I domains in uninterrupted hcpG ORFs change the tertiary structure of HepG ............................................................. 58
HepG is rapidly evolving in diverse H. pylori isolates ............................. 59
Biological relevance of HepG in H. pylori growth and AGS cell infection ...... 60
Non-neutral evolutionary dynamics of hcpC .......................................... 64
Biological significance of HcpC adaptive evolution ............................. 68
Growth kinetics of hcpC single mutant and hcpC-hcpG double mutant ............................................................. 68
HcpC and HcpG paralogs are redundant and contribute additively to relative fitness of *H. pylori* strain G27MA in AGS cell infection ..................69

Role of HcpC and HcpG in surface translocation of HspB ..................72

HcpC and HcpG dependent modulation of HspB surface expression requires the cellular infection.................................................................79

Summary-I......................................................................................80

Role of HepC during *H. pylori* Growth and Infection......................81

Differential regulation of hepC expression in diverse *H. pylori* isolates in AGS cell infection model..........................................................81

HepC contributes significantly to the fitness of *H. pylori*................82

HepC likely targets the host cytoskeletal machinery during late infection in AGS cell culture model of infection........................................84

Genetically diverse *H. pylori* strains differentially dysregulate cytoskeletal regulators during an early infection of AGS cell line infection........87

Summary-II....................................................................................91

DISCUSSION AND FUTURE DIRECTIONS........................................92

REFERENCES....................................................................................102

APPENDIX.........................................................................................115

CURRICULUM VITAE......................................................................119
LIST OF FIGURES

Figure 1: Multiple episodes of positive selection in \textit{H. pylori} \textit{slr} gene family expansion ................................................................. 9

Figure 2: Construction of insertion and deletion alleles by assembly of three fragments with overlapping ends ........................................................... 25

Figure 3: Strategy for knocking out \textit{hcpC} homolog in \textit{H. pylori} strain 26695 StrR. 28

Figure 4: Strategy for knocking out \textit{hcpG} homolog in \textit{H. pylori} strain G27MA .... 31

Figure 5: SLR domain architecture in encoded proteins of \textit{H. pylori} \textit{slr} genes, \textit{hcpC} and \textit{hcpG} in the available \textit{H. pylori} genomes ............................................. 51

Figure 6: Genetic organization of \textit{hcpC} in the available \textit{H. pylori} genomes ........... 52

Figure 7: \textit{hcpG} rapidly evolves in diverse \textit{H. pylori} isolates ........................................................................................................ 55

Figure 8: \textit{Indels} pattern seen among HcpG homologs in diverse \textit{H. pylori} isolates ... 57

Figure 9: HcpG variants differ in their tertiary structures ........................................... 58

Figure 10: Biological relevance of HcpG during \textit{H. pylori} growth and infection ..... 63

Figure 11: Non neutral evolutionary dynamics of \textit{hcpC} ........................................... 66

Figure 12: Growth and relative fitness dynamics of G27MA – \textit{hcp} mutants ......... 70

Figure 13: Role of G27MA HcpC and G27MA HcpG in \textit{H. pylori} surface translocation of HspB .......................................................... 74

Figure 14: Dynamics of CagA and MAPK-YT in G27MA – AGS infection .......... 76
Figure 15: Synthesis of HspB is not affected in G27MA – hcp mutants ............................................................................................... 78

Figure 16: Cellular infection dependent surface translocation defect of HspB in G27MA – hcp mutants ............................................................................ 80

Figure 17: hepC appears to be relevant biologically, and required during H. pylori infection .............................................................................................. 83

Figure 18: HepC likely targets the host cytoskeletal machinery during late infection in AGS cell culture model of infection ............................................................................................................................... 86

Figure 19: Differential dysregulation of cytoskeletal regulators following H. pylori infection with diverse H. pylori strains ................................................................................................................................. 90

Figure 20: Evolution of stable, non-reciprocal genetic redundancy by diversifying selection following duplication and divergence in H. pylori Sel 1-like gene family ................................................................................................................................. 94

Figure 21: Causes and consequences of H. pylori molecular evolution ................................................................................................................................. 100
LIST OF TABLES

Table 1: Parameters for FACS analysis..........................................................47
Table 2: Primers used in the study...............................................................48
Table 3: Strain specific distribution of \( hcpG \) in the available \( H. pylori \) genomes...50
Table 4: Maximum-likelihood parameters of selection pressures acting on \( H. pylori \) \( hcpG \) codons............................................................60
Table 5: Maximum-likelihood parameters of selection pressures acting on \( H. pylori \) \( hcpC \) codons............................................................67
INTRODUCTION

I. Historical aspects of Helicobacter pylori

*H. pylori* is a gram-negative, helical, flagellated, microaerophilic bacterium; it was first identified and isolated from human stomach by B. J. Marshall and R. Warren in 1982 [159, 160], for which they were awarded a Nobel prize in Medicine, in 2005. The bacterium belongs to class *Epsilon - proteobacteria*, family *Helicobacteraceae*, and before being grouped as genus *Helicobacter*, it was classified as genus *Campylobacter* [161]. Studies have shown that humans have been colonized by *H. pylori* for at least 60,000 years, and that as ancient humans migrated out of Central Africa and inhabited different geographic regions, the bacterium had coevolved with its human host [1, 2].

II. Epidemiology and clinical outcome of *H. pylori* infection

*H. pylori* inhabits the gastric mucosa of more than half of world’s population, usually colonizing human stomachs in childhood and persisting throughout the life, thus suggesting effective management and perhaps even exploitation of host responses [3]. Transmission usually occurs locally within families or within small populations through oro-faecal route [4, 5]. In developing countries, the prevalence can be as high as 80-90%, where as in industrialized nations, it ranges between 10-50% [6]. The infection can take multiple courses in its progression. Most people infected with *H. pylori* never develop symptomatic disease, 10-15% develop peptic ulcer disease (gastric and
duodenal ulcers), approximately 1% develop gastric adenocarcinoma, and a small group of patients develop gastric MALT lymphoma [7]. To date, \textit{H. pylori} is the only type I definitive human bacterial carcinogen, estimated to be responsible for 5.5% of all human cancer cases, and up to 8% of all non-Hodgkin lymphoma [8, 9]

Another striking feature of \textit{H. pylori} infection is the wide geographical variance seen in the nature and severity of clinical outcome of the disease. Incidence of gastric cancer in Japan is approximately seven-fold higher than in the US among infected persons [10, 11], and is even rarer in South Asia (India) [10]. Similarly, duodenal ulcers are far more common than in many other geographic regions [11, 12]. Peptic ulcers are rare among infected Greenland Eskimos [13] and Australian Aborigines [14], relative to that in mainstream US and European populations [15]. Although multiple factors like human genetics, diet, and infections by other pathogens that affect responses to \textit{H. pylori} infection [16, 17] can contribute to these trends, an important role could be attributed to the genetic diversity of \textit{H. pylori} itself.

III. Genetic diversity in \textit{H. pylori}

\textit{H. pylori} is an extremely diverse species [18, 19], and there is no single \textit{H. pylori} strain that is "typical" for the species as a whole. Great genetic diversity, population subdivisions, and rapid evolvability are hallmarks of \textit{H. pylori} populations. \textit{H. pylori} population genetic structure can be classified as "panmictic", with very little evidence of clonality or epidemic spread [20]. However, observed "panmixia" is local, and is superimposed on strong geographic differences in predominant genotypes [21-26]. Phylogenetic studies of \textit{H. pylori} housekeeping gene sequences and insertion sequences
revealed strong geographic clustering, wherein types of alleles from different geographic regions (e.g. East Asia, Europe, and Africa) are each distinct, and not overlapping [24-26].

Even greater geographic differences are seen in the virulence-associated cytotoxin antigen-A (cagA) and vacuolating toxin A (vacA) genes [24, 27-31]. Their encoded proteins each interact with target cells and disrupt different sets of normal cellular signal transduction pathways, with strengths and specificities that seem to vary geographically [32]. For example, East-Asian and Western-type CagA proteins differ most markedly in sequence in the domain responsible for phosphorylation (EPIYA motif) and resulting interaction with host SHP-2 phosphatase, an intracellular regulator of cell proliferative, morphogenetic and motility signaling pathways [33, 34]. Similarly, a highly active variant of the vacA toxin gene termed s1, m1 (s: region encoding signal peptide; m: region encoding toxin that determines cell type specificity of VacA toxin) predominates in Japanese isolates, whereas the nontoxicogenic s2, m2 type is relatively common in the West [29, 31]. An intermediate form designated s1, m2 is common in coastal China [35].

Strong geographic clustering was also found in the functionally active middle region of H. pylori adhesin babA (blood group antigen binding adhesin A) [22]. Adherence of H. pylori to gastric mucosa is important for its long-term survival in gastric niche, and contributes significantly to the risk of gastric disease. These geographic differences could reflect genetic drift, or more likely types of selection pressures imposed by host physiologies that predominate (d) in the various human populations, either currently or centuries ago [17]. Given this reasoning, it seems logical that additional H. pylori genes whose products interact with host components might exhibit equivalent geographic
differences, also affecting how they interact with cognate host factors, which might influence clinical outcome of the gastric disease.

Thus, what drive the extraordinary genetic diversity and geographic subdivisions in \textit{H. pylori} populations? Usually, genetic diversity in populations arises through accumulation of point mutations, recombination and genetic exchange. \textit{H. pylori} strains: 1) have high recombination and mutation rates, which can be ascribed to a lack of \textit{mutHLS} like pathway for DNA mismatch repair [36], 2) \textit{H. pylori} are naturally competent for DNA transformation, and can acquire genetic material from other \textit{H. pylori} strains or even from other species in its niche [36], and 3) \textit{H. pylori} have extensive non-randomly distributed DNA repeat sequences that facilitate frequent intragenomic recombination, resulting in deletion or duplication of intervening DNA fragments [37]. Superimposed on these, the extraordinary chronicity of infection along with the barriers to gene flow due to geographic isolation of ancestral \textit{H. pylori} populations cause considerable genetic drift, and thus genetic differentiation of various \textit{H. pylori} subpopulations. Such genetically isolated subpopulations are more likely to “adapt” to variations in local environments [25, 42], and such adaptations could potentially explain the geographic variance found in the nature and severity of infection. However, is there a signature of such adaptations in the population genetic structure of \textit{H. pylori}?

IV. \textbf{Population genetic structure of \textit{H. pylori}: role of positive selection}

In general, genetic drift may allow subpopulations to explore adaptive landscapes and respond to environmental or other changes, then allowing natural selection for particular mutant or recombinant types to help fine-tune genotypes [38]. An effective
way to map selection pressures for protein coding genes is to contrast the rates at which synonymous (silent; \( d_s \)) and nonsynonymous (amino acid altering; \( d_N \)) mutations are fixed in the population [39]. The ratio \( d_N/d_s \) (= \( \omega \)) indicates whether an amino acid change is unaffected, inhibited or promoted by natural selection. Since most synonymous substitutions have no or very little effect on fitness, \( d_s \) is often equated to the rate of neutral nucleotide substitution and hence provides a benchmark against which to measure if \( d_N \) is accelerated or diminished by selection. Under neutral evolution, \( d_N \) is assumed to be equal to \( d_s \) (\( \omega = 1 \)) suggesting a relaxed functional constraint. Functionally critical genes (e.g. housekeeping genes, genes responsible for metabolic functions) are expected to show very low \( d_N \) (\( \omega < 1 \); negative or purifying selection) [39]. However, in certain genes, nonsynonymous mutations are in excess (\( \omega > 1 \); positive Darwinian selection or diversifying selection), because they provide an advantage in a given environmental context, and such substitutions are more likely to be selected to change the activity or structure of the encoded protein [39, 40].

The first study that supported the notion that \emph{H. pylori} subpopulations adapt in response to the differences in local host physiologies was the study on \emph{H. pylori} adhesin, \emph{babA} [22]. \emph{H. pylori} binds to fucosylated histo-blood group antigens (Lewis b, LeB) by an outer membrane protein BabA, which is expressed by most disease causing \emph{H. pylori} strains [41]. Strong geographic partitioning was found in BabA binding affinities to its cognate LeB receptors. \emph{babA} alleles can be classified as generalist and specialist [22]. Generalist alleles (> 95% of \emph{H. pylori} strains) confer binding to fucosylated blood group antigens A, B, and O [22]. However, in Amerindian populations where the predominant host blood group is O, 60% of \emph{H. pylori} strains in these populations bound only to blood
group antigen O (specialists) [22]. Given that, diversifying selection ($\omega = 3.5$) had contributed significantly to $babA$ divergence, it is plausible that the BabA protein had undergone adaptations in response to human population specific selective pressures [22].

Signatures of positive selection ($\omega > 1$) were also evident in the evolutionary history of $H. pylori$ slr (sel 1 like repeat) gene family expansion [42]. Sequence analysis of six of the nine known slr genes ($hp0160$, $hp0211$, $hp0235$, $hp0519$, $hp0628$ and $hp1117$) from representative East Asian, European, and African strains revealed that all but $hp0628$ had been subject to positive selection, with different amino acids often being selected in different geographic regions [42]. Most striking was a divergence of Japanese and Korean alleles of $hp0519$, with Japanese alleles having undergone particularly strong positive selection ($\omega > 25$), whereas Japanese and Korean alleles of other slr and housekeeping genes were intermingled [42]. Homology-based structural modeling localized most residues under positive selection to SLR protein surfaces where they would potentially interact with host components [42]. Rapid evolution of certain slr genes in specific $H. pylori$ lineages was interpreted as reflecting geographic isolation favoring adaptive divergence of bacterial proteins driven by selection for fine tuning of host responses [42].

V. Eukaryotic like Sel1-like repeat (slr) containing gene family in $H. pylori$

Sel1-like repeat (SLR) motif was first identified in a Caenorhabditis elegans extracellular receptor protein, Sel-1 (sel: suppressor-enhancer of lin), which is a key negative regulator of the Notch pathway and regulates cell proliferation, cell fate specification, differentiation, cell death, and endosomal sorting [43-46]. Deregulated
Notch activity is oncogenic in many cases, including gastric cancer [47, 48]. Some eukaryotic SLR proteins are known to function as adaptor proteins for the assembly of membrane-bound macromolecular complexes [49-52]. Studies on bacterial SLR proteins suggest that they can aid in the adaptation of bacteria to different eukaryotic hosts [52-57]. Based on the evidence available so far, the SLR motif is absent in archa and viruses suggesting that SLR domains have been acquired by horizontal gene transfer between bacteria and eukaryotes [58, 59] [60]. It is also possible that SLR domains have evolved in the last common ancestor of eukaryotes and bacteria playing a role in cellular differentiation [60].

The *H. pylori* slr gene family has eight members ('slr' genes) in sequenced *H. pylori* genomes [61, 62] and they belong to cluster of orthologous group (COG) 0790. Their encoded proteins are likely secreted and contain a variable (2-8) number of highly degenerate 34 amino acid Sel1-repeats [63]. Six of the nine *H. pylori*’s SLR proteins are rich in cysteines, and had been designated as ‘Helicobacter cysteine rich proteins’ (Hcp) [64, 65]. A disulfide bond bridges the α-helices of each SLR repeat, which is a unique feature of Hcps [60]. Although the exact in vivo function of *H. pylori* SLR proteins is not currently known, some Hcps bind β-lactam compounds [64, 66], which suggests possible interactions with immunomodulatory peptidoglycan fragments, that could affect the innate immune response [67]. High antibody titres against four SLR proteins [Hp0211 (HcpA), Hp0235 (HcpE), Hp0336 (HcpB), and Hp1098 (HcpC)] were found in sera of *H. pylori*-infected people [65], indicating in vivo expression and immune recognition. Furthermore, recombinant HcpA elicited IL12-dependent IFN-γ secretion in a naïve mouse splenocyte model [67], and induced differentiation of freely circulating monocytes
into adherent macrophages [68]. It was also shown that HP1117 elicited protective antibodies during a mouse infection [69]. *H. pylori* SLR protein, HepC (Hp0519), interacts with the multifunctional human cytoskeletal proteins Ezrin and Vinculin, and different geographic variants of HepC differ in their binding affinity to Ezrin [Putty K *et al.*, *in preparation*]. Such interaction differences likely manifest in altered Ezrin-dependent signal transduction and may influence the progression and outcome of gastric disease in different geographic regions.

Among the genomes of other related Epsilon-proteobacteria, hepatocarcinogenic *Helicobacter hepaticus* has six ORFs, and the relatively nonpathogenic *Campylobacter jejuni*, *Campylobacter lari*, *Campylobacter upsaliensis* and *Wolinella succinogenes* each have only one ORF corresponding to *slr* gene homologs [70-72]. This suggests an *H. pylori* specific expansion of *slr* gene family, perhaps reflecting a gastric niche specific adaptive role.

VI. **Expansion of *H. pylori slr* gene family by gene duplication**

A duplication event leads to relaxed selective constraints on one copy, such that it is free to diverge. In the absence of a new selective constraint such as new environment, the duplicated copy may accumulate deleterious mutations and suffer functional loss and ultimately get degraded [74-81]. However, in the presence of a positive selective pressure, randomly generated mutations by genetic drift may encode a functional variant that is critical for survival under new conditions [73-82].

Taken together, the evidence of diversifying/positive selection during the evolutionary history of a gene family is strongly suggestive of functional divergence
among related members. Maximum likelihood phylogenetic analysis using codon-based models of sequence evolution revealed that *H. pylori sir* gene family expansion was driven by diversifying selection ($\omega > 1$) (Fig. 1), implying functional divergence among *sir* paralogs [42]. Superimposed on this, individual *sir* genes (*hp0160, hp0211, hp0235, hp0519, and hp01117*) evolve rapidly, often in distinct human-host lineages, suggesting a key role in mediating host-pathogen interactions [42].

![Figure 1: Multiple episodes of positive selection in *H. pylori sir* gene family expansion [42].](image-url)
A structure-based alignment of *H. pylori* SLR proteins from strains 26695, J99, and HPAG1 was derived using EXPRESSO. A corresponding *sir* gene family sequence alignment was derived manually. An initial ML tree, used as input for selection analysis, was generated assuming the TrN + I + model of sequence evolution. Phylogeny shown above was estimated under the FR model implemented in PAML version 3.14. Branches that experienced positive selection during their evolution are indicated by dotted lines, and dN, dS, and ω values are indicated. A ω ratio = 0 indicates branches that only accumulated nonsynonymous mutations during their divergence. Scale indicates number of substitutions per codon.

VII. **Host cytoskeletal dysregulation following *H. pylori* infection**

An important feature of cultured gastric epithelial cells infected with *H. pylori* is the development of a humming bird phenotype characterized by loss of cell-cell contacts, cell elongation, and eventual acquisition of motility [83, 84]. Regulated cell motility can mediate embryonic morphogenesis, tissue repair and regeneration, whereas deregulation in this highly integral process can lead to disease progression in cancer [84]. Important biological events associated with cell migration are breaking apart of intercellular complexes and cytoskeletal rearrangements, leading to elongation of the leading edge, adhesion of this protrusion to the matrix, movement of the cell body, and release of the trailing edge of the cell [84-86]. Focal adhesions (FA) and actin cytoskeleton are the important structures that regulate cell motility. FAs are comprised of a transmembrane α-β integrin heterodimer, the extracellular domain of which binds to extra cellular matrix proteins and anchors actin cytoskeleton on the cytoplasmic side of the membrane, mediating various intracellular signaling pathways [87]. Integrin activation induces recruitment and stimulation of a variety of signaling and adapter proteins (FAK, Src) that
target and activate members of Rho GTPases, which thereby regulate actin rearrangements that control cell elongation and migration [83].

Injection of CagA into epithelial cells, its phosphorylation at FAs, and subsequent deregulation of downstream signaling pathways affecting cell spreading, cell movement, and cell survival requires binding of H. pylori protein CagL to the β1 integrin receptor of epithelial cells [88-91]. Phosphorylated CagA binds to: 1) Shp-2 (Src homology 2 domain-containing tyrosine phosphatase), activating Shp-2/Rap1/B-Raf/Erk signaling pathway, inducing elongated cell morphology [92-98]; 2) CrkII (v-crk sarcoma virus CT10 oncogene homolog), activating the DOCK180/Rac1/WAVE/Arp2/3 pathway, leading to the actin cytoskeletal rearrangement [99]. H. pylori infection also leads to the activation of Rho GTPase Rac1, activating WAVE (WASP family verprolin-homologous protein) and the Arp2/3 (actin-related protein 2/3) complex, leading to formation of broad sheet-like protrusions containing a network of branching actin filaments, lamellipodia, which are usually found at the leading edge of migrating cells [100, 101].

However, CagA independent mechanisms of actin cytoskeletal rearrangements in H. pylori infection were also reported [102-105], suggesting a role for other, as yet unidentified secreted H. pylori factors in the pathogenesis of this bacterium. Understanding the pathogenesis of H. pylori and identification of H. pylori population specific virulence determinants are areas of active research. Key questions that remain to be fully answered are the following:

1. What are the molecular signals and evolutionary forces that enable H. pylori to establish a chronic infection in the gastric niche in the face of continuous innate and adaptive immune responses?
2. What are the *H. pylori* induced signaling events that initiate gastric carcinogenesis?

3. Why clinical outcomes of *H. pylori* infection vary geographically?

4. Are there any clear-cut markers that can confidently predict clinical disease outcome?
SPECIFIC AIMS

Emerging data from recent studies suggest a possible role for proteins encoded by the sir genes in mediating and / or managing H. pylori - host interaction. However, the molecular and evolutionary dynamics of strain-specific sir genes remain to be fully characterized. Strain-specific genes often contribute to strain-specific traits. Based on this premise I posit that strain-specific sir genes in H. pylori genomes likely contribute importantly to strain-specific features during H. pylori's extraordinarily chronic infection. Thus, a first goal of my thesis is to characterize the molecular evolutionary dynamics of hcpC and hcpG, each demonstrating unique strain-specific features, in order to establish a framework within which to further understand their role in H. pylori pathogenesis.

Gene duplication is an important driving force for biological innovation in any species. Our data showed H. pylori genome specific expansion of sir gene family, driven by strong diversifying selection [42], and led to the identification of the paralogous genes, hcpC and hcpG. However, the evolutionary forces that contributed to the origin, maintenance and preservation of these paralogous genes in natural H. pylori populations is not known. Furthermore, the influence of these paralogous genes upon H. pylori - host interaction remains to be determined.

Phylogenetic and protein structure analyses had shown that H. pylori sir gene hp0519 (hepC) alleles in different H. pylori lineages evolve rapidly at different rates thereby leading to selection of different amino acids in different populations, that amino
acids under positive selection in affected HepC lineages were exposed on HepC surfaces, and that positive selection altered specific amino acids in the Japanese HepC lineage and helped drive its divergence from the Korean HepC lineage; whereas in all the other slr genes, Japanese and Korean alleles were invariably intermingled (42). Ongoing work in the lab had identified that HepC interacts directly with the multifunctional human cytoskeletal protein Ezrin, and that different geographic variants of HepC differ in their binding affinity to Ezrin. Thus, I hypothesized that geographically distinct H. pylori strains likely differ in their ability to deregulate host cytoskeletal dynamics.

Taken together, although available data strongly implicate selection as a potent force during H. pylori's evolutionary history it remains unclear how selective forces might have shaped H. pylori - host interactions. Thus, the specific aims of my dissertation are the following:

Aim 1: To characterize the molecular and evolutionary dynamics of strain-specific, paralogous slr genes, hcpC and hcpG.

Aim 2: To determine how hcpC and hcpG each affect H. pylori growth and fitness, and identify their role in mediating H. pylori - host interaction.

Aim 3: To determine the functional relevance of HepC during H. pylori - host interaction.

Aim 4: To quantify H. pylori population-specific variations in human cytoskeletal dysregulation.
MATERIALS AND METHODS

I. Helicobacter pylori culture and maintenance

A. Standard H. pylori culture, growth and maintenance

Bacteria were grown on plates of selective brain heart infusion (BHI) agar (Difco, KS) supplemented with 7 % (v / v) defibrinated horse blood (Cleveland Scientific, OH), isovitalex (BBL Medium enrichment for Fastidious Microorganisms, BD, France), H. pylori selective supplement, Dent (vancomycin 10 mg / L, cefsulodin 5 mg / L, polymyxin B 2,500 U / L, trimethoprim 5 mg / L and amphotericin B 7.5 mg / L) (Oxoid Ltd, Basingstoke, Hants, England). Erythromycin (10 µg / ml), streptomycin (10 µg / ml), and/ or chloramphenicol (10 µg / ml) were added to the agar as needed to select for mutant colonies. Cultures were incubated at 37°C, for 3-4 days in a GasPak jar (BBL Microbiology Systems, Cockeysville, Md.) with a micro aerobic gas mixture (CampyPak, BBL Microbiology Systems, Cockeysville, MD) composed of 5 % oxygen, 10 % carbon dioxide, and 85 % nitrogen [106]. Strains were maintained as frozen stocks by scraping bacterial growth from agar plates with a sterile loop in BHI broth (Difco, KS) with 15 % glycerol, aliquoted in 1ml freezer tubes, and stored at -74°C.

B. Culture of H. pylori in liquid media

H. pylori was grown in liquid medium using BHI broth supplemented with 1% (v / v) Isovitalex, 1% H. pylori selective supplement, and 10% fetal bovine serum (FBS)
Liquid medium in 50ml tissue culture flask was inoculated with the starting bacterial suspension (OD$_{600}$ 0.05/ml and incubated at 37°C for 30 min in CO$_2$ incubator. Then the flasks were transferred to GasPak jars and incubated at 37°C with shaking (125 rpm) for a maximum of 56hrs [106].

II. DNA Extraction, PCR-conditions, and DNA sequencing.

A. Genomic DNA isolation

*H. pylori* genomic DNA was isolated from confluent cultures on agar plates by the hexadecyltrimethylammonium bromide (CTAB) method [109, 110]. Briefly, 1 X 10$^8$ bacteria were collected and washed twice in 1X TE buffer. To the cell pellet, SDS and proteinase K were added to a final concentration of 0.5% (v / v) and 100 µg / ml, respectively and the mixture was incubated at 37°C for 1 hr. 80 µl of CTAB / NaCl solution was then added and incubated for 20 min at 65°C. Proteins were then removed using phenol / chloroform / isoamyl alcohol method. DNA was precipitated with 0.6 volumes of 100% v / v isopropanol, washed twice with 70% ethanol to remove residual CTAB, dried and suspended in 1X TE buffer. Genomic DNA was stored at -20°C until required.

B. PCR conditions and DNA sequencing

i) General PCR conditions:

Specific PCR reactions were carried out in 25 µl mixtures containing 5 to 10 ng of DNA, 1 U of Taq polymerase (Biolase; Midwest Scientific, St. Louis, Mo.), 1.5 mM of MgCl$_2$ (Biolase; Midwest Scientific, St. Louis, Mo.), 0.8 pmol of each forward and
reverse primers, and each deoxynucleoside triphosphate at a concentration of 0.1 mM in a standard buffer for 30 cycles with the following cycling parameters: denaturation at 94°C for 30 s, annealing at a temperature appropriate for the primer sequence (generally 54°C) for 30 s, and DNA elongation at 72°C for an appropriate time (1 minute per kb).

ii) *hcpC* and *hcpG* amplifications:

To amplify complete nucleotide sequences of *hcpC* and *hcpG* homologs, and to detect their chromosomal conservation in genetically diverse *H. pylori* isolates, one set of primers was designed located within *jhpl023* and *jhpl025*, the genes flanking *jhpl024* (the J99 homolog of *hcpC*), and in *jhpl1436* and *jhpl1438*, the genes flanking *jhpl1437* (the J99 homolog of *hcpG*), respectively. To help prevent primers from being designed in segments with high frequencies of mutations, *jhpl023* was aligned with *hpagl035* from strain HPAG1, and *jhpl025* was aligned with *hpagl_1037* from HPAG1 and *hp1099* from 26695. Similarly, *jhpl1436* was aligned with *hpg27_1468*, and *jhpl1438* was aligned with *hpg27_1470* from strain G27MA. This alignment was done in MegAlign (DNASTAR package; Lasergene Inc., USA). Once the flanking genes were aligned, primers were designed in regions of the sequence conserved in the alignment. The resulting primers, *hcpCF1* (Primer #1; Table 2) located in *jhpl023* and *hcpCR1* (Primer #2; Table 2) located in *jhpl025* amplified an approximately 1643 base pairs long segment; *hcpGF1* (Primer #3; Table 2) located in *jhpl1436* and *hcpGR1* (Primer #4; Table 2) located in *jhpl1438* amplified 988 bp long segment, in strain J99. To increase sequence redundancy coverage of *hcpC* sequences missing 30 or more base pairs due to a failure of the sequences from both primers to overlap, a third internal primer was designed.
(1098Fseq) (Primer #5; Table 2). This primer was designed 200 base pairs within *hp1098* at a site found to be well conserved when several of the first sequences to be analyzed were aligned with *hp1098*. This primer was then used to sequence *hcpC* homologs that showed incomplete or ambiguous data. Since no amplification was observed in approximately 50% of *H. pylori* strains tested using *hcpG* flanking primers, a second set of primers (hcpGF2 and hcpGR2) (Primer #8 & 9; Table 2) was designed within *jhp1437* to amplify a 750bp product at sites found to be well conserved when several sequences obtained initially were analyzed. To confirm either the absence of *hcpG* homolog in the genome or its chromosomal rearrangement, hcpGF2 and hcpGR2 primers were then used to amplify those *H. pylori* strains that showed no amplification or a smaller amplicon than expected fragment size using primers designed in the flanking genes of *jhp1437*. These primers were then used to amplify *hcpC* (by Sarah Marcus under my guidance as part of her undergraduate honors thesis) and *hcpG* homologs from 166 strains of *H. pylori* from Spain, Japan, Korea, Gambia, South Africa, India, Peru, and Shima (Peruvian Amazon). These strains had been isolated from patients with gastric complaints that underwent a diagnostic endoscopy with informed consent. Amplification of these genes and the specificity of PCR amplification were confirmed using 1% agarose gel electrophoresis. Only amplifications that yielded a single specific product were processed further.

iii) DNA sequencing: *hcpG* and *hcpC* sequence determination:

The DNA concentration of PCR products was determined using a BioPhotometer (Eppendorf) to ensure quality products for sequencing. 10 µl of the amplified product was
then placed in two 96-well plates for sequencing. Each plate was paired with a second plate containing either 10 μl of primer hcpCF1 or hcpCR1, and hcpGF1 or hcpGR1; these primers are from either end and were used to sequence hcpC and hcpG homologs, respectively. The plates were then shipped to High-Throughput Genomics Unit, Seattle, WA, where the PCR products were purified and sequenced on both strands using an ABI377 automated sequencer.

III. Computational Biological Analyses.

A. DNA Sequence Data Processing and Management

Sequences from both forward and reverse strands were assembled and edited in Seqman (DNASTAR, Lasergene Inc., WI). hcpC and hcpG sequences from the available H. pylori genome sequences in Genbank were included in the respective alignments to ensure proper alignment, and to properly trim the sequence data to only the sequence of the gene of interest. Seqman was also used to view the sequence data to ensure that the sequence data was not contaminated, due to nonspecific amplification for example, or incorrectly interpreted by the computer. Any errors were noted and corrected if the data clearly showed a peak for a different nucleotide than the one indicated. Those sequences that had poor data, possibly from contamination, were removed from the group, and sequencing was repeated.

B. Multiple Sequence Alignments

Complete sequences of the hcpC, and hcpG homologs were then translated into amino acids and aligned initially in MegaAlign using Clustal W and default settings. A
preliminary phylogenetic tree was then reconstructed in MEGA version 5 using the Neighbor-joining method, and bootstrap analysis with 1000 replicates. The Mega5 alignment was saved as a nexus file for use in PAUP*4b10 (http://paup.csit.fsu.edu/). The alignment was then used to create a neighbor-joining tree used as input for MODELTEST, version 3.7 (http://darwin.uvigo.es/software/modeltest.html). MODELTEST determines the model of DNA evolution that best describes the given data. This test is important for choosing an appropriate model, because although more complex tests add more parameters to make a model more realistic, additional parameters also leave more room for errors. Therefore a model should not be chosen if it is more complex than the data provided merits.

C. Phylogenetic Reconstruction

The MODELTEST program [111] was used to find the best model by measuring the likelihood that a null model fits the given data compared to the likelihood that an alternative model fits given the same data. The first test in the program, hierarchical likelihood ratio tests (hLRTs) compares the likelihoods of nested models, or models which build off one another, becoming more complex with more parameters as the program continues the comparisons [112]. These models can easily be compared for goodness of fit using a chi square test and the model with the highest likelihood of a pair is used for the next comparison through a series of more complex models, until the model of best fit is determined. The second test run in MODELTEST is the Akaike information criterion (AIC). This test can function without consecutive models being nested, and it again tests for goodness of fit. It also adds a penalty for unnecessary parameters. Both of
these tests will not only determine the model of best fit, but will give the estimated values for each of the parameters used in that model.

Once the model of best-fit was determined, a maximum likelihood (ML) phylogeny was reconstructed under this model by using a combination of heuristic searches and branch swapping to further optimize the likelihood score and substitution parameters. ML generates a tree that most likely explains the given data. To find which tree is most likely, a heuristic search was conducted starting with a single tree, and altering it in a single way. If the new tree is more likely than the previous tree, the new tree was kept and the old tree was discarded, and the process was repeated sometimes over 100,000 times. If the original tree is more likely, it would be kept until an improved tree was found. This process was continued to search through trees until the most likely tree was found. Although a heuristic search is not an exhaustive search through all possible trees, leaving the possibility that only suboptimal tree is given as the most likely tree, this approach is necessary due to the exponential number of possible trees and the many weeks that would be needed to sort through all of them computationally.

The significance of the observed phylogenetic groupings was assessed by a bootstrap analysis performed with 1,000 replicates under the distance optimality criterion, while incorporating the ML-optimized model and parameters. Bootstrapping was performed in order to estimate sampling error by sampling from within the provided data creating pseudoreplicates, which is used to generate a new tree. This method is then repeated, here 1000 times, and a bootstrap consensus tree displaying the most frequent splits labeled at their nodes with their frequencies. These frequencies help determine the precision of the methods used to estimate the tree with the given data, providing an
estimate of confidence in the phylogeny. Phylogenetic trees were visualized with TreeView version 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html), and were edited in Adobe photoshop CS8 software.

D. Analyses of Selection Pressures

The selective pressures operating on \textit{hcpC} and \textit{hcpG} were measured using an ML method that takes into account the sequence phylogeny and assesses the fit to the data of various models of codon evolution that differ in how $\omega$ varies across the sequence or across the phylogeny. Site specific models (SSMs) in codon-based analysis (M0, M1, M2, M3, M7, M8, and M8a) assume a single $\omega$ for all branches of the tree, but allow $\omega$ to vary among individual codon sites, thereby providing a measure of heterogeneity in selection pressures acting across the gene sequence [113, 114]. Positive selection was inferred when codons with $\omega$ of $>1$ were identified and the likelihood score (-lnL) of the codon substitution model in question was significantly higher than the likelihood of a nested model that did not take positive selection into account. The probability that a specific codon belonged to the neutral, negative, or positively selected class was calculated using empirical Bayes methods implemented in PAML version 4.14 (http://abacus.gene.ucl.ac.uk/software/paml.html). Multiple runs, assuming different initial $\omega$ and $\kappa$ values, and different models for estimating equilibrium codon frequencies (to be calculated from the average nucleotide frequencies at the three codon positions tables (F3X4) or used as free parameters), were analyzed for \textit{hcpC}, and \textit{hcpG} to verify the convergence optima for each model.
E. Domain Architecture Analyses

Identification and annotation of domain architectures of HcpC, and HcpG variants was performed using a Simple Modular Architecture Research Tool (SMART), PFAM and Signal IP. Domains were extensively and confidently annotated with respect to phyletic distributions, functional class, tertiary structures and functionally important residues. Parameters are given in Appendix I.

F. Homology Modeling

Homology modeling of HcpG variants was performed using a web based version of Modeller v 9.8, webmod. Available HcpB and HcpC crystal structures were used as template models for the analysis.

IV. Genetic Engineering of H. pylori.

A. General Method for Introducing Marked Deletions in H. pylori

The general principle for generating H. pylori slr gene deletions in this study was described previously [115]. Briefly, a streptomycin resistant (str$^R$) allele of rpsL (str$^S$) was generated by PCR by introducing A-to-G changes at each of the two sites most frequently responsible for streptomycin resistance (codons 43 and 88; Lys to Arg in each case) [116]. This allele was then used to transform str$^S$ 26695 H. pylori strain to generate a str$^R$ 26695. Genomic DNA of str$^R$ 26695 was then used to transform G27MA (cell culture adapted H. pylori strain) to generate str$^R$ G27MA. rpsl-erm or rpsl-cat cassettes conferring streptomycin susceptibility (rpsL gene from Campylobacter jejuni, codes for ribosomal protein S12, a dominant allele for streptomycin sensitivity) and erythromycin
or chloramphenicol resistance, respectively were then used to generate PCR based constructs where the flanking genes of antibiotic cassettes were homologous to genes flanking the targeted gene deletion. These constructs were generated by assembling individual PCR products which have overlaps of $\geq 20$ bp at the ends of fragments to be joined together, which in turn, result from the design of PCR primers used in amplification (sewing - PCR) [117, 118] (Fig. 2). Primers 2 and 5, each on the inside ends of their respective fragments were designed not only to match the sequence at the end of the fragments amplified, but also to have approximately 20 bp overlap with fragment B. Primer 2 began at its 5'end with a segment that is the reverse complement of primer 3, and primer 5 begins with a segment that is the reverse complement of primer 4. These overlaps are essential for assembly of fragments A, B, and C. Once the primers were designed, standard PCR amplification was used to separately generate PCR products A, B, and C. These three products were then combined and amplified using PCR with primers 1 and 6. The overlapping segments at the ends of fragments A and B and at the starts of fragments B and C allow the fragments to be “sewn” together as amplification occurs, resulting in a final contiguous PCR product ABC (Fig. 1) [115]. Such constructs were then used to transform str$^R$ strains to select for erythromycin or chloramphenicol resistance and screen for str$^S$ mutant colonies.
Figure 2: Construction of insertion and deletion alleles by assembly of three fragments with overlapping ends [115].

Half arrows indicate the positions of the primers. Segments A and C represent DNA segments flanking the locus to be deleted or the site of insertion (depending on needs of experiment). Segment B represents (i) the *erm* (resistance) gene used initially to replace the *mdaB* locus; (ii) the *rpsL* streptomycin susceptibility gene from *C. jejuni* that was inserted just upstream of *erm* in a strain carrying *erm* in place of *mdaB*; or (iii) the *rpsL,erm* cassette, which can be moved to many loci [115] (with permission from DEB).

B. Engineering Δ*hcpC* mutation in *H. pylori* strain G27MA.

The overall strategy used for generating Δ*hcpC* mutation was similar to that described above with a few modifications as noted below. Three sets of primers were designed. The first set for amplification included 1098A1n and 1098A2n as primers 1 and 2, respectively, for the amplification of a 437bp long fragment A. Fragment A began at the end of *hp1098* with hp1098A1n (primer # 8; table 2) starting 804 bp within the gene and ending at the start of primer hp1098A2n (primer # 9; table 2) 368 bp within the gene. This fragment was not positioned in the gene downstream of *hp1098* (*hp1097*), as is
described in the general procedure above, because homologs to *hp1097* are not present in all genomes from which homologs to *hp1098* may need to be amplified to transform back into the knockout. Fragment B, as described above, was the *rpsL-erm* cassette and primers *rpsL-F-I* (primer # 10; table 2) and *erm-R* (primer # 11; table 2), corresponding to primers 3 and 4, respectively, were used for its amplification. Their sequences are also the reverse complements for the overlapping sequences found at the 5’ ends of 1098A2n, which overlaps with *rpsL-F-I*, and 1098C5n, overlapping with *erm-R*. The final set of primers, 1098C5n (primer # 12; table 2) and 1098C6n (primer # 13; table 2), corresponding to primers 5 and 6, amplified a 440 bp long fragment C. This fragment began at the start of primer 1098C5n, 20 bps within *hp1098* and extended to the start of 1098C6n placed 276 bps from the start of *hp1099*. Primers were synthesized by Integrated DNA technologies, Inc. IA. Using the primers described above, fragments A, B, and C were each amplified using a standard PCR. Fragments A and C were run with an annealing temperature of 55°C and fragment B with an annealing temperature of 58°C. All of the PCR products were purified by spin column purification. 3 μl of each of the products from the first round of amplification were used in an assembly PCR using primers 1098A1n and 1098C6n with an annealing temperature of 54°C. Similarly, 3 μl of product B and the PCR products A and C from the alternative primers were used for two more assembly PCRs with annealing temperatures of 55°C and 58°C. Poor amplification of the PCR assembly led to an attempt at a two part assembly, which has been found to be successful when the single three fragment assembly fails [119]. For this PCR assembly, 3 μl of alternative fragment A and fragment B were amplified together, and alternative fragment C and fragment B were amplified together to form two combined
fragments: AB and BC. 3 μl of each of these PCR products were then used for a second PCR assembly to give the final ABC assembly which contained the rpsL-erm cassette within hp1098 (Fig. 2). The final assembly was then used to transform H. pylori strain 26695 StrR which was grown overnight and re streaked in the center of a fresh plate in the morning. The transformation was completed by adding 10 μl of the ABC assembly to the cells and mixing them with a loop at the center of the plate. These cells were then grown overnight and the following day they were streaked on a fresh plate containing erythromycin to select for EryR and presence of the rpsL-erm cassette. After growing several days, individual EryR colonies were picked using toothpicks and streaked in a line on corresponding erythromycin- and streptomycin-containing plates. Colonies observed on the erythromycin plate and not on the streptomycin plate were then streaked across individual erythromycin plates so that the cells could be used to make frozen stocks and streptomycin plates as a final check for StrS. Representative colonies that passed the antibiotic screening as both EryR and StrS were then tested by PCR using primers 1098A1n and 1098C6n. Colonies showing PCR products matching the expected PCR product size of 2388bp were considered to be successful knockouts with the rpsL-erm cassette inserted in and replacing part of hp1098 (hcpC). The 26695-ΔhcpC genomic DNA was then used to transform H. pylori strain G27MA StrR to generate a G27MA-ΔhcpC-strS and ermR strain. This hcpC mutant strain in Hp26695 was generated by Sarah Marcus in the lab of Dr. Douglas E. Berg as a part of her undergraduate honors thesis.
Figure 3: Strategy for knocking out hcpC homolog in *H. pylori* strain 26695 StrR.

Primer 2 overlaps (hanging line) with primer 3 and 5 overlaps with 4 so that fragments A and C, on either side of *hp1098* overlaps with the ends of B in the final assembly. Light gray filled boxes represent non-coding sequences. Fragment B (*rpsl-erm*) confers streptomycin susceptibility and erythromycin resistance. Genomic DNA of *H. pylori* strain 26695-*hcpCΔ* was then used to transform G27MA-StrR *H. pylori* strain to generate G27MA:*hcpCΔ-StrS and ermR.

C. Engineering ΔhcpG mutation in *H. pylori* strain G27MA

Three sets of primers were designed. The first set for amplification included hcpGAF and hcpGAR as primers for the amplification of a 700 bp long fragment A. Fragment A began at 24 bp of *jhp1436* and ends at the beginning of *jhp1437*. Fragment B, was the *rpsL-cat* cassette and primers rpsL-F-1 and cat-R, were used for its
amplification. Their sequences are also the reverse complements for the overlapping sequences found at the 5' ends of hcpGAR, which overlaps with rpsL-F-1, and hcpGCF, overlapping with erm-R. The final set of primers, hcpGCF and hcpGCR would amplify a 640bp long fragment C. This fragment began at the start of primer hcpGCF, at the end of *jhp1437* and extended to the 640 bp into *jhp1438*. Primers were synthesized by Integrated DNA technologies, Inc. IA. Using the primers described above, fragments A, B, and C were each amplified using a standard PCR. All of the PCR products were purified by spin column purification. 3 µl of each of the products from the first round of amplification were used in an assembly PCR using primers hcpGAF and hcpGCR with an annealing temperature of 54°C. Poor amplification of the PCR assembly led to the alternate procedure as described above where fragments AB and BC were generated first and then “sewn” together resulting in ABC. However, even this procedure resulted in poor amplifications. As a result, this procedure was used with few modifications. An alternate set of four primers were then designed. Primer hcpGAR was replaced with hcpGAR2 (primer 2) (primer # 15; Table 2) with the same sequence as hcpGAR along with an added restriction site of *Ecorl*-GAATTC at its 5’ end. Primer rpsL F2 (primer 3) (primer # 16; table 2) had restriction site GAATTC added to its 5’ end. Primer catR2 (primer 4) (primer # 17; table 2) had restriction site GCGGCCGC-NotI at its 5’ end which matches the 5’ end of primer hcpGCF2 (primer 5) (primer # 18; table 2). Forward primer of fragment A (hcpGAF) (primer 1) (primer # 14; table 2) and reverse primer of fragment C (hcpGCR) (primer 6) (primer # 19; table 2) were the same as before. First, fragments A, B, and C were amplified using alternate set of primers. PCR products were then purified using Qiagen PCR purification kit. 1 µg of each fragment was then taken and restriction
enzymes EcoRI and NotI were then added to the mix along with 1X digestion buffer making a final volume of 20 μl. Restriction digestion was then performed at 37°C for 3hrs. Digested products were then purified and ligated with the addition of T4 DNA ligase in a 30 μl total reaction volume and incubated overnight at 16°C. Primers hcpGAF and hcpGCR were then used to amplify fragment ABC from the ligated mix and the PCR product was run on a 1 % agarose gel to confirm the presence of ABC fragment at the expected size 2950 bp. The PCR construct was then used to transform H. pylori strain G27MA strR which was grown overnight and restreaked in the center of a fresh plate in the morning. The transformation was completed by adding 10 μl of the ABC assembly to the cells and mixing them with a loop at the center of the plate. These cells were then grown overnight and the following day they were streaked on a fresh plate containing chloramphenicol to select for CatR and presence of the rpsL,cat cassette. After growing several days, individual CatR colonies were picked using toothpicks and streaked in a line on corresponding chloramphenicol- and streptomycin-containing plates. Colonies observed on the chloramphenicol plate and not on the streptomycin plate were then streaked across individual chloramphenicol plates so that the cells could be used to make frozen stocks and streptomycin plates as a final check for StrS. Colonies that passed the antibiotic screening as both CatR and StrS then had their genomes amplified by PCR using primers hcpGAF and hcpGCR. Colonies showing PCR products matching the expected PCR product size of 2950bp were considered to be successful knockouts with the rpsL-cat cassette inserted in place of hcpG (Fig. 3). The PCR products were finally sequenced to check for any unwanted mutations in flanking regions other than the restriction sites that were introduced immediately before and after the rpsl-cat cassette.
Figure 4: Strategy for knocking out hcpG homolog in *H. pylori* strain G27MA.

Fragment A has restriction site GAATTC at its 3' end which matches with restriction site at 5' end of fragment B (red overhang). Fragment C has restriction site GCGGCCGC at its 5' end which matches with restriction site at 3' end of fragment B (green overhang). Fragment B (rpsl-cat) confers streptomycin susceptibility and chloramphenicol resistance. Restriction digestion with enzymes *EcoR1* (red), *Not1* (green); ligation with T4 DNA ligase and subsequent PCR with primers 1 and 6, yields knockout assembly ABC, which was then used to transform G27MA-StrR *H. pylori* strain, to generate G27MA-hcpGΔ-StrS and catR. The same strategy was used to
generate *H. pylori* strain G27MA-HcpG 6XHis where fragment B (rps-l-cat) was replaced with hcpG:6XHis. Lanes 1, 2, 3, 4, 5, 6, and 7 in the gel have molecular weight marker, fragments A, B (hcpG::His), C, ABC (B-rps-l-cat), ABC (B-hcpG::His), respectively.

D. Engineering HcpG::6XHis insertion in G27MAΔhcpG derivative

The strategy described in section IVC was also used to generate *H. pylori* strain G27MA-HcpG::6X His, expressing HcpG with a 6X Histidine tag at its C - terminus. Primers hcpGBF and hcpGBR (Table 2) were designed to amplify hcpG homolog in *H. pylori* strain G27MA with a 6X His tag at its 3’ end. This was achieved by adding a 6X His tag sequence to the 5’ end of hcpGBR sequence. Primers hcpGBF (primer # 20; table 2) and hcpGBR (primer # 21; table 2) also contained restriction sites for EcoRI and NotI at their 5’ ends so that fragment B (hcpG::6X His) matched with fragments A and C at its 5’ end and 3’ end, respectively. Using fragments A, B, and C, construct ABC (2250 bp) was generated as described above and the construct was then used to transform G27MA-ΔhcpG CatR strain, which was grown overnight and restreaked in the center of a fresh plate in the morning. The transformation was completed by adding 10 µl of the AB2C assembly to the cells and mixing them with a loop at the center of the plate. These cells were then grown overnight and the following day they were streaked on a fresh plate containing streptomycin to test for CatS and presence of the hcpG::His. After growing several days, individual StrR colonies were picked using toothpicks and streaked in a line on corresponding streptomycin and chloramphenicol containing plates. Colonies observed on the streptomycin plate and not on the chloramphenicol plate were then streaked across individual streptomycin plates as a final check for StrR so that the cells could be used to make frozen stocks. Expression of functional HcpG::6XHis in the
G27MA-HcpG::6XHis strain was confirmed by monitoring expression of HcpG in *in vitro* grown G27MA-HcpG::6XHis by reverse transcription PCR, and by detecting HcpG::6X His by immuno blotting with an anti-His antibody (described below).

**E. Engineering ΔhcpCΔhcpG mutations in *H. pylori* strain G27MA**

Genomic DNA was extracted as described in earlier section from the *H. pylori* G27MA-ΔhcpC strains generated above. 10 μl of this genomic DNA was used to transform G27MA-ΔhcpG CatR strain which was grown overnight on a chloramphenicol plate and restreaked in the center of a fresh plate in the morning. Genomic DNA was mixed with bacteria on the plate and mixed with a loop at the center of the plate. These cells were then grown overnight and the following day they were streaked on a fresh plate containing both erythromycin and chloramphenicol to select for ErmR and CatR respectively (and absence of *hcpC* and *hcpG*, respectively). After growing several days, individual ErmR and CatR colonies were picked using toothpicks and streaked in a line on corresponding chloramphenicol-erythromycin and streptomycin containing plates. Colonies observed on the chloramphenicol-erythromycin plate and not on the streptomycin plate were then streaked across individual chloramphenicol-erythromycin plates as a final check for ErmR and CatR so that the cells could be used to make frozen stocks. Colonies that passed the antibiotic screening as both CatR and ErmR then had their chromosomal regions amplified by PCR using primers 1098A1n and 1098C6n and, hcpGAF and hcpGCR. Colonies showing PCR products matching the expected PCR product size of 2388bp (with primer pairs 1098A1n and 1098C6n) and 2950 bp (with primer pairs hcpGAF and hcpGCR) were considered to be successful knockouts with the
rpsL-erm and rpsL-cat cassettes inserted in respectively and replacing part of hcpC and whole hcpG, respectively in H. pylori strain G27MA.

F. Engineering ΔhepC mutations in H. pylori strains G27MA and 26695

hepC deletions in H. pylori strains 26695 and G27MA were generated as described in section IVA and were kindly provided by Dr. Douglas E. Berg and Dr. Ozge Darka (Washington University School of Medicine, St. Louis, MO).

V. Cell Culture and Antibodies

A. Culture and maintenance of cell line, AGS

ATCC CRL 1739, a human gastric adenocarcinoma epithelial cell line (AGS) was cultured and maintained in antibiotic free DMEM-high glucose (Sigma), supplemented with 10% heat inactivated fetal bovine serum (GIBCO). Cells were grown and maintained in 50 ml tissue culture flasks at 37°C in a humidified atmosphere of 5% CO2.

B. AGS cell culture for assaying infection dynamics, including cytoskeletal assays

48 hrs before infection, AGS cells were seeded in 6-well plates at a density of 0.5x10^5 cells / well after a viability count in haemocytometer, using the trypan blue exclusion assay. 12 hrs before infection, the cells were maintained in serum free medium. For the infection assays, bacteria were harvested and washed in PBS (pH 7.4), and were diluted to a multiplicity of infection (MOI) of 100 i.e., 100 H. pylori / AGS cell. To synchronize the infection, the plates were centrifuged for 10 min at 1000 X g using a SORVALL Legend RT centrifuge and incubated for indicated time intervals at 37°C in a
humidified atmosphere of 5% CO₂. To quantify the AGS cell scattering phenotype 6 hrs post infection, images of infected and uninfected AGS cells were taken at 20X magnification in a Nikon Eclipse TE-2000U fluorescent microscope. Cell extensions were measured using Metamorph software and extensions measuring over 40 μm from the center of the cell were considered “scattered” or “hummingbird” phenotype [120]. A total of 100 cells were counted in each field and a minimum of three such fields were counted in each experiment. Uninfected AGS cells were used as controls wherever necessary.

C. AGS cell culture for assaying bacterial growth and fitness

24 hrs before infection, AGS cells were seeded in 6-well plates at a density of 10⁵ cells / well, after doing a viability count in haemocytometer using trypan blue exclusion assay. Since H. pylori growth dynamics was monitored here, AGS cells were not serum starved before the infection. Instead, wells were supplemented with 20% FBS during infection. For the infection, desired bacterial strains were harvested in PBS (pH 7.4), washed with PBS, and were diluted to a multiplicity of infection (MOI) of 100. To synchronize the infection, the plates were centrifuged for 10 min at 1000 X g using a SORVALL Legend RT centrifuge and incubated for indicated time points at 37°C in a humidified atmosphere of 5% CO₂.

D. Antibodies used in the study

Primary antibodies used in this study are: anti-CagA (goat IgG) sc-34039, anti-VASP (mouse IgG) sc-46668, anti-Helicobacter pylori HSP (mouse IgG) sc-57779
[purchased from Santacruz Biotechnology, Santa Cruz, CA]; anti- phospho-Map kinase kinase (rabbit IgG) M-76783, anti-HIS (mouse IgG), anti-FLAG (mouse IgG), anti-GST (mouse IgG), anti-tubulin (mouse IgG) [kindly provided by Dr. Swathi Arur, MD Anderson Cancer Center, TX]. Secondary antibodies used in this study were: horseradish peroxidase conjugated secondary (HRP - mouse and HRP - goat) [kindly provided by Dr. Swathi Arur, MD Anderson Cancer Center, TX], Goat polyclonal Secondary Antibody to Mouse IgG - H&L (FITC) ab6785, Donkey polyclonal Secondary Antibody to Goat IgG - H&L (FITC) ab6881, Donkey F(ab')2 polyclonal Secondary Antibody to Rabbit IgG - H&L (PE) ab7007, Goat polyclonal Secondary Antibody to Mouse IgG - H&L (TR) ab6787 [purchased from Abcam, MA or kindly provided by Dr. Palaniappan Sethu, Dept. of Bioengineering, University of Louisville, KY].

VI. Immunoblotting

Crude cell lysates or purified protein preparations were separated on 5-10% Tris-HCl polyacrylamide gels and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) with the Bio-Rad Mini-Protean system. Membranes were blocked in TBS-T (140 mM NaCl, 2.7 mM KCl, 25 mMTris-HCl, 0.1% (V/V) Tween) with 5% non-fat dry milk for 1h at room temperature, and washed 3 times with TBST. Membranes were then incubated in desired primary antibodies overnight at 4°C. After washing thrice with TBST, primary antibodies were detected using horseradish peroxidase conjugated secondary antibodies and visualized by Western Lightning Chemiluminescence Reagent (Peirce) according to the manufacturer's instructions, using Versadoc 4000. Between
blotting procedures, the membranes were stripped for 30 min at 50°C in stripping buffer (62.5 mM Tris-HCl, pH 6.7, 100 mM 2-mercaptoethanol, 2% SDS).

**VII. Growth Kinetics and Fitness Assays.**

**A. In vitro Growth Kinetics and Fitness Assays**

i) *In vitro* growth dynamics of engineered HpG27MA derivatives:

To assess the *in vitro* growth dynamics of G27MA WT and its *hcp* mutants (*hcpC* mutant or *hcpG* mutant or *hcpC, hcpG* double mutant) tested, bacterial strains were grown on BHI agar plates for 3 days as described in section IA with selective antibiotics. Individual *H. pylori* strains were then inoculated and grown in BHI broth as described in section IB. Serial dilutions of bacterial aliquots from the inoculums were plated on selective plates: G27MA WT - streptomycin, G27MAΔhcpC - erythromycin, G27MAΔhcpG - chloramphenicol, G27MAΔhcpC : ΔhcpG - erythromycin and chloramphenicol to obtain 0 hr bacteria counts. At 6 hr (lag growth phase), 24 hr (log growth phase), 48 hr (stationary phase), and 56 hr (late stationary phase), bacterial aliquots from the cultures were taken, serial dilutions made and plated on selective plates for counting colony forming units (CFUs). The plates were incubated for 4-5 days as described in section IB. At the end of incubation, colonies were counted and the number of bacteria was expressed as the number of CFU/ml. The experiment was repeated three more times and statistical significance between groups was calculated using a Student's *t*-test.
ii) Competition and relative fitness assays with HpG27MA derivatives and G27MA WT:

To assess the relative fitness of G27MA WT and each of the *hcp* mutants, (*hcpC* mutant or *hcpG* mutant or *hcpChcpG* double mutant) *in vitro*, each of the mutant was competed with the WT. Bacterial strains were grown on BHI agar plates for 3 days as described in section IA. Each BHI broth tube was inoculated with G27MA str<sup>R</sup> along with either *hcpC* mutant or *hcpG* mutant or *hcpChcpG* double mutant with a starting bacterial OD<sub>600</sub> of 0.05 / ml and cultured as given in section IB. Serial dilutions of bacterial aliquots from the initial inoculums were plated on selective plates-G27MA WT-streptomycin, *G27MAΔhcpC*-erythromycin, *G27MAΔhcpG*-chloramphenicol, *G27MA-ΔhcpC; ΔhcpG* - erythromycin and chloramphenicol to obtain 0 hr bacteria counts. At time intervals 12 hr and 56 hr, bacterial aliquots from the cultures were taken, serial dilutions made and plated on selective plates (streptomycin for WT, erythromycin, chloramphenicol, erythromycin and chloramphenicol depending on the mutant tested for fitness) for CFUs. For example, when fitness of *G27MAΔhcpC* strain was tested against G27MA WT, at each time interval serial dilutions from the bacterial culture were plated on both erythromycin plates and streptomycin plates. BHI plates were incubated for 4-5 days as described in section IB. At the end of incubation, colonies were counted and the number of bacteria was expressed as the number of CFU / ml. The competitive Index (CI) was then calculated as the ratio of mutant CFU / ml to wild type CFU / ml at each time interval / ratio of mutant CFU / ml to wild type CFU / ml in the inoculums. CI value less than 1 indicates that wild type is favored over the mutant; a value more than 1 indicates that mutant is favored over the wild type. The CI is equivalent to fitness (F) of a given
bacterial strain relative to the WT [121]. The median reduction in fitness (S) was computed as \((1 - CI)\). The experiment was repeated three more times and statistical significance between groups was calculated using a Student’s t-test.

B. Growth Kinetics and Fitness Assays in AGS cell culture infection model

i) Growth dynamics of engineered HpG27MA derivatives in AGS infection model:

AGS cells were seeded in 6-well plates at a density of \(10^5\) cells / well, 24hrs before infection as described in section VC. Each well was infected with a single desired bacterial strain (G27MA WT, or G27MA\(_{A\Delta hcpC}\), or G27MA\(_{A\Delta hcpG}\), or G27MA\(_{A\Delta hcpC: A\Delta hcpG}\), or G27MA\(_{A\Delta hcpC}\)) at MOI of 100. To synchronize the infection, the plates were centrifuged for 10 min at 1000 X g using a SORVALL Legend RT centrifuge and incubated at 37°C, 5% CO\(_2\). Serial dilutions of bacterial aliquots from the initial inoculums were plated on selective antibiotic resistant plates as described above to obtain 0 hr bacteria counts. At the end of time intervals 6 hr and 24 hr, the whole well was scraped gently and mixed well. Serial dilutions were prepared and aliquots were then plated on selective antibiotic resistant BHI plates for counting. The number of bacteria was expressed as CFU / ml. Each experiment was repeated three more times and statistical significance between groups (WT and mutant) was calculated using a Student’s t-test. (Note: CFUs of each growth dynamics experiment performed in this study are given in the Appendix).
ii) Competition and Relative Fitness Assays with HpG27MA derivatives and G27MA WT in AGS infection model:

To assess the relative fitness of G27MA WT and each of the $slr$ mutants, ($hcpC$ mutant or $hcpG$ mutant or $hcpC$, $hcpG$ double mutant or $hepC$ mutant) in an AGS cell infection, each of the mutant was competed with the WT. The experimental strategy used in VIIIB(i) was used with the only exception being that each AGS well was infected with the two bacterial strains. For example when the fitness of $G27MA-hepCd$ strain was tested against G27MA WT, each AGS cell well was infected with both the strains with a MOI of 100 each. At the end of each time interval, 6 hr and 24 hr, serial dilutions from the bacterial culture were plated on both chloramphenicol (for selecting $hepC$ mutant) and streptomycin (for selecting WT) plates. Plates were incubated and CI was calculated as described in VIIA(ii). Each experiment was repeated three more times and statistical significance between groups (WT and mutant) was calculated using a Student's $t$-test.

VIII. Reverse-transcription PCRs.

A. RNA extraction, purification and preparation for RT-PCR analyses

For RNA extraction, cells were lysed in buffer RLT (Qiagen), homogenized and purified using RNeasy mini kit (Qiagen) following manufacturer's instructions. RNA was dissolved in 30 µl of RNAase free water and checked for integrity using Agilent Bioanalyzer 2100. Determining RNA integrity is a critical step in gene expression analysis. Thus we determined RNA integrity and quality using three different measures, which included electropherogram analysis, determination of 16S:23S rRNA ratio and the RNA integrity number (RIN). RIN is a powerful new tool and is expressed as a scale that
ranges from 1 – 10. A RIN value closer to 10 indicates greater RNA integrity, and thus potentially high reproducibility for high throughput gene expression analysis (eg., microarrays). These analyses showed that the extracted RNA had RIN numbers that ranged from 7.5 to 9.2 (Appendix V). cDNA was then synthesized using the purified total RNA and the cDNA synthesis kit (Qiagen, USA), and used as template for reverse-transcription PCR and real-time PCR. Briefly, DNA was removed from 500 ng-1 μg RNA by adding 2 μl of 7X g.DNA wipe out buffer, and incubated at 42°C for 10 minutes. Then, 1 μl of Quantitect Reverse transcriptase, 1 μl of random primer mix or desired primers (1 μM), 4 μl of 5X Quantiscript RT Buffer was added and incubated at 42°C for 15 min. Reverse transcriptase was then inactivated by incubating at 95°C for 3 min. The generated cDNA was used immediately or stored at -20°C.

B. Qualitative Assays for detecting slr gene transcripts, in vitro and in AGS cell culture infection model

To detect expression of hcpG in genetically diverse H. pylori strains: J99 (European), 26695 (European), JS7 (Japanese), G27MA (cell-culture adapted), K17 (Korean), R7 (South African), Pecan32 (Peruvian), and S46 (Spanish) in vitro, bacterial growth was collected for each strain grown on BHI agar plate; RNA was then extracted and converted to cDNA as described in section VIII A. A set of primers were designed in the internal region of hcpG (hcpGF-cDNA and hcpGR-cDNA), which is conserved among all the strains tested, and which amplified a 250 bp product. As a positive control, a primer set was designed within the recA gene (recAF and recAR) of H. pylori, amplifying a 206 bp product. To detect expression of hcpG in diverse H. pylori strains
after an AGS cell culture infection, AGS cells were infected with the indicated \textit{H. pylori}
strains as described in section VB. 6 hr post infection, RNA was extracted as described
before and converted to cDNA. As a positive control for AGS infection, a primer set
gapdhF and gapdhR was designed in the internal region of \textit{gapdh} gene amplifying a 278
bp product. Since genomic DNA contamination of cDNA can produce false positive
results, RNA alone was used as a template in the PCR reactions as a negative control.
Using the designed primers PCR reactions were performed as described in section IIB(i)
using cDNA as template. To detect the expression of \textit{hepC} in \textit{H. pylori} strain G27MA, a
set of primers were designed in the internal region of \textit{hepC} (hepCF-cDNA and hepCR-
cDNA) amplifying a 150bp product using procedures described above.

\section*{C. Quantitative-RT PCRs for determining \textit{slr} gene expression levels \textit{in vitro} and in
AGS cell culture infection models}

To quantify the transcript expression level of \textit{slr} genes in this study grown \textit{in vitro}
and in AGS cell culture infections, experiments were set up as desired and cDNA was
synthesized as described above section VIIIIB. Since comparison of gene expression in
real-time PCR assay assumes that the all the primer sets used in the analysis have equal
amplification efficiency, all the primer sets were designed using ABI primer express
software version 3.0, so that they should have the same amplification efficiency. Primers
jhp1024F and jhp1024R, jhp1437F and jhp1437R, jhp468F and jhp468R were used to
amplify \textit{hcpC}, \textit{hcpG}, and \textit{hepC} homologs, respectively. The generated cDNA was diluted
fivefold with RNase free water. All real time PCR reactions were performed in 20 \mu l
mixture containing 1 / 10 volume of cDNA preparation (2 \mu l of 1:5 diluted c DNA), 1X
Power SYBR green PCR master mix (10 μl) (Applied Biosystems, Foster City, CA, USA), 5 μM of each forward and reverse primer (1 μl) and making up the reaction volume to 20 μl with water. RNA quantitations were performed in Applied Biosystems Step-One thermocycler using the following PCR conditions: 95°C for 10 min followed by 95°C for 15 s and 60°C for 1 min for 40 cycles. Appropriate no-RT and non-template controls were included in each reaction plate and melting curve analysis was performed at the end of each run to confirm the specificity of the reaction. The concentration was determined by the comparative C_T method (threshold cycle number at the cross-point between amplification plot and threshold) and values were normalized to ureA/recA. Negative and positive values were considered as down-regulation or up-regulation of expression of genes of interest, respectively, represented by a minimum of two-fold difference.

D. Pathway-specific PCR arrays

The Human cytoskeleton regulations pathway-specific PCR arrays (PAHS-088A) developed by SABiosciences permits simultaneous interrogation of gene expression of 84 genes controlling the intracellular scaffolding’s biogenesis, organization, polymerization, and depolymerization.

AGS cells were infected with a representative European (J99, and G27MA), Japanese (JS7), Amerindian (SHI470) H. pylori strains, for 3 hrs and 6 hrs. Next, RNA was extracted from AGS cells, equal amount (500 ng) of RNA was used in all the arrays to convert to cDNA as described above and quantitative PCRs were conducted using the pathway-specific PCR array with appropriate controls for human DNA contamination.
(higXIA), housekeeping genes (β2M, hgprt, rpl13A, g3pd and β-actin), and multiple positive and negative controls (supplied by the manufacturer & given in the appendix). The expression of cytoskeletal regulators was then compared with an uninfected control to identify genes that were differentially up or down regulated by ΔΔCt method, following infection with diverse H. pylori strains. The analysis was performed through a integrated web-based software package for the PCR Array System which performs all quality checks and ΔΔCt based fold-change calculations from the uploaded raw threshold cycle data obtained for each condition. Fold up or down regulations were depicted as scatter plots with a cut off of two fold change.

IX. Fluorescence Activated Cell Sorting Analyses

A. Determination of Variation in Heat Shock Protein B (HspB) surface expression by H. pylori G27MAWT and ΔhcpC and ΔhcpG derivatives in vitro

To detect the variation in HspB surface expression among G27MA WT and hcp mutants grown in vitro, indicated strains were grown in BHI broth as described in section IB. BHI broths inoculated with hcpC mutant, hcpG mutant, and hcpC, hcpG double mutant contained 10 μg / ml of erythromycin, chloramphenicol, and erythromycin with chloramphenicol, respectively, to provide selection. At 3 hr, 24 hr, and 56 hr post infection, 3 ml of bacterial cultures were aliquoted, spun at 3500 rpm for 10 min and washed twice with 1X PBS (pH:7.4). Cells collected were immediately fixed using 4% paraformaldehyde at room temperature for 15 min. The cells were then washed twice with 1X PBS and stored immediately at 4°C. Once the cells were fixed, all the washing steps were done at a speed not more than 2000 rpm until a cell pellet is visible (or for a
maximum of 10 min). After the cell pellets from all the desired time points were obtained, surface staining of HspB was conducted by skipping the cell permeabilization step. Anti-HSP primary antibody was then added to the cell suspension at a final concentration of 1 µg/ml and incubated on ice for 30 min, followed by washing twice with wash buffer (1X PBS + 0.2% BSA). Secondary antibody conjugated with texas red (TR) fluorophore was then added to cell suspension at a final concentration of 0.5 µg/ml and incubated for 30 min on ice, in dark. Cells were then washed twice with 1X wash buffer and analyzed in a BD FACS Calibur™ Flow Cytometer (BD Biosciences) using channel FL4 for detecting emitted fluorescence from $10^4$ cells/sample. Unstained bacteria were used to optimize the scatter (Forward scatter-FSC and Side scatter-SSC) and to establish background fluorescence. After obtaining mean fluorescence for each sample, the data was analyzed in WinMDI software version 2.9 to obtain mean geometric fluorescence values and depicting fluorescent intensities as histograms or dot plots. Parameters used in the detection of fluorescence were given in Table 1.

**B. Determination of Variation in Heat Shock Protein B (HspB) surface expression by *H. pylori* G27MAWT and ΔhcpC and ΔhcpG derivatives in AGS cell culture infection model**

After infecting AGS cells (as described in section VB) with either the hcpC mutant or hcpG mutant or hcpC, hcpG double mutant for 3hr, 6hr, 12hr, and 24hr, AGS cells were washed twice with 1X PBS to remove unattached bacteria and detached using 2mM EDTA. Cells collected were immediately fixed using 4% paraformaldehyde at room temperature for 15 min. Once the cells from all the desired time points were
obtained, surface staining of HspB was conducted and analyzed as described above in section IXA. Unstained AGS cells with adhered bacteria were used to optimize the scatter and to establish back ground fluorescence. The experiment was done in triplicate and a student t-test was used to calculate statistical significance between experimental groups.

C. Determination of CagA, VASP and activated MAPK expression analyses in the context of AGS infection model

To ensure the specificity of HspB dynamics in *hcp* mutants and *G27MA WT H. pylori* strain, expression dynamics of CagA, VASP, and activated MAPK were monitored as controls. CagA expression is used as a control for equal bacterial inoculums. Increase in host cell VASP expression and activation of MAPK are hallmarks of *H. pylori* infection. Thus, they were used as controls for monitoring host cell dynamics. CagA, VASP, and activated MAPK antibodies used in this study were raised against three different hosts: anti goat, anti mouse, and anti rabbit, respectively. After infecting AGS cells (as described in section VB) with either the *hcpC* mutant or *hcpG* mutant or *hcpC*hcpG double mutant for 3 hr, 6 hr, 12 hr, and 24 hr, AGS cells were washed twice with 1X PBS to remove unattached bacteria and detached using 2 mM EDTA. Cells collected were immediately fixed using 4% paraformaldehyde at room temperature for 15 min. Once the cells from all the desired time points were obtained, they were permeabilized using 0.01% Triton-X (Sigma Aldrich, MO) for 3 min at room temperature. Cells were immediately washed twice with 1X wash buffer. The three primary antibodies were then added to each of the tubes containing permeabilized AGS cells at a final concentration of 1 μg/ml and incubated on ice for 30 min, followed by
washing twice with wash buffer (1X PBS + 0.2% BSA). Secondary antibodies conjugated with either texas red (anti VASP) or phycoerythrin (PE) (anti-activated MAPK), or FITC (anti-CagA) fluorophores were then added to cell suspension at a final concentration of 0.5 μg / ml and incubated for 30 min on ice, in dark. Cells were then washed twice with 1X wash buffer and analyzed in a BD FACS Calibur Flow Cytometer (BD Biosciences) using channel FL1 (FITC), channel FL2 (PE) and channel FL4 (TR) for detecting emitted fluorescence from $10^4$ cells/ sample. Unstained AGS cells with adhered bacteria were used to optimize the scatter and to establish back ground fluorescence. Data was then analyzed in WinMDI 2.9 as described above.

Table 1: Parameters for FACS analysis.

<table>
<thead>
<tr>
<th></th>
<th>α-HSP</th>
<th>α-CagA/VASP/pSer MAPK</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSC</td>
<td>E00</td>
<td>E00</td>
</tr>
<tr>
<td>SSC</td>
<td>387</td>
<td>408</td>
</tr>
<tr>
<td>FL1</td>
<td>412</td>
<td>364</td>
</tr>
<tr>
<td>FL2</td>
<td>150</td>
<td>400</td>
</tr>
<tr>
<td>FL3</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>FL4</td>
<td>150</td>
<td>819</td>
</tr>
</tbody>
</table>

FSC - forward scatter, SSC – side scatter, FL1, FL2, FL3, FL4 – fluorescent channels 1, 2, 3, and 4, respectively to detect fluorescence emitted from FITC, PE, and TR conjugated fluorophores, respectively.
Table 2: Primers used in the study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer name</th>
<th>Sequence 5'-3'</th>
<th>Location</th>
<th>Product size</th>
<th>Intended use (added sequences and restriction sites italicized and underlined)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>hcpGRI</td>
<td>5'AGGACAAAGGGTTTGTT</td>
<td>bp1023:747-769</td>
<td>140bp Amplification of hcpG with flanking gene fragments</td>
<td>This study</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hcpGF2</td>
<td>5'TCAAAAAAGCGGTTTTAGGG</td>
<td>bp1436:274-293</td>
<td>98bp Amplification of hcpG with flanking gene fragments</td>
<td>This study</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hcpGF-c DNA</td>
<td>5'-AGAAGGCTGGGGCTCATTTG</td>
<td>bp1438:26-42</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hp1437F</td>
<td>5'-AGGCGTGGCAAAGGATGA</td>
<td>bp1437 11-31</td>
<td>500bp Amplification of fragment A for hcpG' knockout</td>
<td>This study</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hcpGCR</td>
<td>5'-TTACAAGATTTAGAGTAAAT</td>
<td>bp1509 20-37</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hcpGAF</td>
<td>5'-GAAGCCTCATTATAAGAAT</td>
<td>bp1509 269-299</td>
<td>500bp Amplification of fragment C for hcpG' knockout</td>
<td>This study</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hcpGR-c DNA</td>
<td>5'-TTACAAGATTTAGAGTAAAT</td>
<td>bp1509 269-299</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hpl098</td>
<td>5'-GTG17TMTCCATAG1TATAAAGCATCCAGCAATGGGTGTCATTTG</td>
<td>bp1098 747-769</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hpl1024</td>
<td>5'-GTTCGCATCACCCATTTGACT</td>
<td>bp1098 747-769</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hpl1098C6</td>
<td>5'-GAAGCCTCATTATAAGAAT</td>
<td>bp1509 269-299</td>
<td>500bp Amplification of fragment C for hcpG' knockout</td>
<td>This study</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hpl1098C5n</td>
<td>5'-GTG17TMTCCATAG1TATAAAGCATCCAGCAATGGGTGTCATTTG</td>
<td>bp1098 747-769</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hpl1098Aln</td>
<td>5'-GTTCGCATCACCCATTTGACT</td>
<td>bp1098 747-769</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hpl1098C5</td>
<td>5'-GAAGCCTCATTATAAGAAT</td>
<td>bp1509 269-299</td>
<td>500bp Amplification of fragment C for hcpG' knockout</td>
<td>This study</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hpl1098C6</td>
<td>5'-GTG17TMTCCATAG1TATAAAGCATCCAGCAATGGGTGTCATTTG</td>
<td>bp1098 747-769</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hpl1098Aln</td>
<td>5'-GTTCGCATCACCCATTTGACT</td>
<td>bp1098 747-769</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hpl1098C5</td>
<td>5'-GAAGCCTCATTATAAGAAT</td>
<td>bp1509 269-299</td>
<td>500bp Amplification of fragment C for hcpG' knockout</td>
<td>This study</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hpl1098C6</td>
<td>5'-GTG17TMTCCATAG1TATAAAGCATCCAGCAATGGGTGTCATTTG</td>
<td>bp1098 747-769</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hpl1098Aln</td>
<td>5'-GTTCGCATCACCCATTTGACT</td>
<td>bp1098 747-769</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hpl1098C5</td>
<td>5'-GAAGCCTCATTATAAGAAT</td>
<td>bp1509 269-299</td>
<td>500bp Amplification of fragment C for hcpG' knockout</td>
<td>This study</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hpl1098C6</td>
<td>5'-GTG17TMTCCATAG1TATAAAGCATCCAGCAATGGGTGTCATTTG</td>
<td>bp1098 747-769</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hpl1098Aln</td>
<td>5'-GTTCGCATCACCCATTTGACT</td>
<td>bp1098 747-769</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hpl1098C5</td>
<td>5'-GAAGCCTCATTATAAGAAT</td>
<td>bp1509 269-299</td>
<td>500bp Amplification of fragment C for hcpG' knockout</td>
<td>This study</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hpl1098C6</td>
<td>5'-GTG17TMTCCATAG1TATAAAGCATCCAGCAATGGGTGTCATTTG</td>
<td>bp1098 747-769</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hpl1098Aln</td>
<td>5'-GTTCGCATCACCCATTTGACT</td>
<td>bp1098 747-769</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Primer used in hcpG' part of the study**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer name</th>
<th>Sequence 5'-3'</th>
<th>Location</th>
<th>Product size</th>
<th>Intended use (added sequences and restriction sites italicized and underlined)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>hpl098F</td>
<td>5'CCAAACCGGATGCTGATTIC</td>
<td>bp1023:747-769</td>
<td>750bp Amplification of fragment C for performing RT-PCR</td>
<td>This study</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hpl098R</td>
<td>5'CAGGACTGACGACCGCCATTTAAA</td>
<td>bp1023:747-769</td>
<td>750bp Amplification of fragment C for performing RT-PCR</td>
<td>This study</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Unique homing gene primers used in the study**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer name</th>
<th>Sequence 5'-3'</th>
<th>Location</th>
<th>Product size</th>
<th>Intended use (added sequences and restriction sites italicized and underlined)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>hpl1024F</td>
<td>5'-CCAAACCGGATGCTGATTIC</td>
<td>bp1024:747-769</td>
<td>275bp Amplification of fragment C for performing RT-PCR</td>
<td>This study</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hpl1024R</td>
<td>5'CCAGACTGACGACCGCCATTTAAA</td>
<td>bp1024:747-769</td>
<td>275bp Amplification of fragment C for performing RT-PCR</td>
<td>This study</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Reference**

This study: The study performed at the laboratory. This study includes the authors' institutional review board approval and ethical considerations. Reference: This study includes the authors' institutional review board approval and ethical considerations.
RESULTS

I. Evolution of Stable Genetic, and Functionally Non–Reciprocal Redundancy
Driven by Positive Selection in Duplicated Sel-1 like Genes of H. pylori.

H. pylori strain – specific sir genes

To determine the distribution and genetic organization of H. pylori strain –
specific sir genes, hcpC, and hcpG in the available complete genomes of H. pylori, we
conducted a BLAST search using the representative homologs in H. pylori strain J99.
These analyses revealed that while hcpC was present in all the available H. pylori
genomes, only four of ten harbored hcpG homologs (Table 3). The prototype HcpC from
H. pylori strain 26695 contained 7 Sel-1 domains with a predicted signal peptide,
whereas encoded proteins of hcpG homologs in the available H. pylori genomes
demonstrated a much more variable domain architecture with alleles containing 4-7 Sel-1
domains and with a predicted signal peptide (Fig. 5). When compared to the other
available H. pylori genomes, genomic location of hcpC is not conserved in H. pylori
genome 26695 (Fig 6). Investigation of the genes upstream of hcpC in available H. pylori
genomes revealed that, except in the genome of Hp26695, the flanking gene (hp1097; short
chain alcohol dehydrogenase) was conserved. Additionally, two genes encoding for
transposases, tnpA (hp1096) and tnpB (at hp1095) were identified 1 kb to 3 kbs upstream
of hcpC, in the genome of strain 26695. It is likely that these transposases encode a
functional two-element insertion sequence of the IS605 family, several of which are
known to be present in *H. pylori* genomes [26] and, which could potentially account for the genetic rearrangement observed in the genome of strain 26695 at the *hcpC* locus. This also suggested that although the *hp1097* homolog may not be in the same chromosomal location with respect to *hp1098* (*hcpC*), it might not have been lost altogether from the 26695 genome. Therefore, I conducted a BLAST (*blastn*) search with *jhp1023* (*J99* homolog of *short chain alcohol dehydrogenase*) sequence against the 26695 genome. This analysis revealed that, *hp0357* was homologous to *jhp1023* and also confirmed that the *hcpC* locus had indeed been subject to genetic rearrangements in strain 26695.

**Table 3: Strain specific distribution of *hcpG* in the available *H. pylori* genomes.**

<table>
<thead>
<tr>
<th>Genome</th>
<th><em>hcpC</em></th>
<th><em>hcpG</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>J99</td>
<td><em>jhp_1024</em></td>
<td><em>jhp_1437</em></td>
</tr>
<tr>
<td>26695</td>
<td><em>hp1098</em></td>
<td>-</td>
</tr>
<tr>
<td>G27</td>
<td><em>hpg27_1039</em></td>
<td><em>hpg27_1469</em></td>
</tr>
<tr>
<td>HPAG</td>
<td><em>hpag1_1036</em></td>
<td>-</td>
</tr>
<tr>
<td>SH470</td>
<td><em>hpsh_05640</em></td>
<td>-</td>
</tr>
<tr>
<td>P12</td>
<td><em>hpp12_1063</em></td>
<td>-</td>
</tr>
<tr>
<td>B8</td>
<td><em>hpb8_406</em></td>
<td><em>hpb8_1690</em></td>
</tr>
<tr>
<td>HP51</td>
<td><em>khp_0998</em></td>
<td>-</td>
</tr>
<tr>
<td>HP52</td>
<td><em>hpkb_1028</em></td>
<td>-</td>
</tr>
<tr>
<td>HPV225d</td>
<td><em>hpv225_1116</em></td>
<td><em>hpv225_1471</em></td>
</tr>
</tbody>
</table>
Figure 5: SLR domain architecture in encoded proteins of *H. pylori* slr genes, *hcpC* and *hcpG* in the available *H. pylori* genomes.

The schematic here shows predicted SLR domains (dotted box), signal peptide motifs (grey box), and their amino acid positions. HcpC domain architecture is identical in all the available *H. pylori* genomes, whereas *hcpG* variants encode proteins with different domain architectures.
Figure 6: Genetic organization of hcpC in the available H. pylori genomes.

hcpC is flanked by 2-keto-3-deoxy-6-phosphogluconate aldolase (eda) and short chain alcohol dehydrogenase (sad) in all the available genomes except H. pylori 26695, where it is flanked by eda and a hypothetical protein (hp1097). Type A is seen in H. pylori genomes J99, P12, G27, HPAG, SHI470, 51, 52 and V225d; type B in B8 and type C in 26695. Genes encoding for transposases (tnpA and tnpB) are found 1kb-3kb downstream hcpC in 26695, which could possibly explain the genetic rearrangement seen.

Genetic rearrangement at hcpC locus is unique to H. pylori strain 26695

Next, I asked if the genetic rearrangement with respect to the hcpC locus was unique to strain 26695 or was also present in the genomes of other diverse H. pylori strains. To determine this, two primers (primers 1 and 2 in Table 2) designed in genes flanking jhp1024 (J99 homolog of hcpC) were used to PCR amplify hcpC homologs in genetically diverse H. pylori strains. 166 out of 166 H. pylori isolates tested variously from Africa (Gambia and South Africa), East Asia (Japan and Korea), India, Peru, Spain, and Shimaa were positive for hcpC locus specific PCR amplification with products of nearly the same size as expected from HpJ99 (1643 bp). This outcome indicated that the hcpC locus rearrangement seen in strain Hp26695 genome was not common in H. pylori strains.
Strain specific distribution of \textit{H. pylori} \textit{slr} gene, \textit{hcpG}

Next, I sought to determine the chromosomal organization and distribution of \textit{H. pylori} strain – specific \textit{slr} gene, \textit{hcpG} in genetically diverse \textit{H. pylori} isolates. To determine this, I used primers (primers 3 and 4 in Table 2) designed in the genes flanking \textit{jhp1437} (J99 homolog of \textit{hcpG}) to PCR amplify \textit{hcpG} homologs in a worldwide collection of 166 \textit{H. pylori} strains. I found that 47\% (79 / 166) of the isolates tested were positive for PCR amplification, although with random size variations deviating from the expected amplicon size (988 bp), when viewed on a 1\% agarose gel (Fig. 7A). Next, I asked if the chromosomal location of \textit{hcpG} was rearranged in the strains that appear to lack \textit{hcpG} (87 / 166). If this were true, it could explain the absence of \textit{hcpG} in these strains tested for its presence using primers designed in the flanking genes. To determine this, I designed primers (primers 6 and 7 in Table 2) in the conserved internal region of \textit{hcpG} homologs and used them to PCR amplify \textit{hcpG} internal fragment in \textit{H. pylori} strains that resulted in no amplification using flanking primers. My results indicated that \textit{hcpG} was indeed absent in 53\% (87 / 166) of \textit{H. pylori} isolates tested.

Unique DNA sequence polymorphisms and pseudogenization of \textit{hcpG}

Next, I asked if the size variations seen in \textit{hcpG} homologs affect the open reading frame (ORF) of their respective encoded proteins. To determine this, full-length PCR products of \textit{hcpG} homologs were sequenced using primers from either end. \textit{hcpG} DNA sequence analysis revealed the following key features: 1) Encoded ORFs of 36\% (28 / 79) of \textit{hcpG} homologs were interrupted by premature stop codons forming truncated proteins (< 100 amino acids) or no protein altogether. Premature stop codons resulted
mostly from a frame shift of the ORF that originated several base pairs upstream of the stop codon (Fig 7B), and also due to base substitutions in a few homologs. 2) In hcpG homologs with uninterrupted ORFs (> 100 amino acids), striking indel (insertions and deletions of nucleotides) polymorphisms were observed (Fig. 8). Taken together, I identified striking genetic variation in hcpG homologs, among diverse H. pylori isolates. This finding was in accord with a previous study where it was shown that hcpG (designated as hsp12 in the study) exhibited high genetic variation [122].
Figure 7: *hcpG* rapidly evolves in diverse *H. pylori* isolates.
(A) Size variations of hcpG homologs in diverse H. pylori isolates. Depicted here is a 1% agarose gel with hcpG homologs amplified from Gambia (1 - 3), Japan (4 - 12), Korea (13 - 19), and Spain (20 - 23). Lane 24 harbors hcpG homolog amplified from H. pylori strain J99.

(B) Pseudogenization in hcpG homologs, as a result of frame shift mutations. Chromatograms shown here depict nucleotide sequences of two different hcpG homologs amplified from Japanese H. pylori strains Hu131 and Hu56. Single base deletion (T-indicated by a star) in Hu56 resulted in a frame shift of Hu56 hcpG homolog, leading to the formation a premature stop codon (TAA), 17 bp downstream of the single base pair deletion. Such frame shift resulted in pseudogenization of hcpG homolog in H. pylori strain, Hu56.

(C) ML phylogeny of HcpG (n = 46) with random geographical distribution of HcpG Sel1 domain variants. The phylogeny was reconstructed assuming the TVM + G substitution model and was optimized to the following parameters using heuristic searches and tree bisection-reconnection algorithm: rate matrix: A→C = 1.8144, A→G = 8.4557, A→T = 0.4947, C→G = 1.2924, C→T = 8.4557, and G→T = 1; base frequencies: A = 0.3623, C = 0.1295, G = 0.2197, T = 0.2885, proportion of invariable sites, I = 0 and gamma distribution shape parameter = 0.3955. Phylogeny is rooted using the outgroup method implemented in PAUP. Bar scale = 0.1 nucleotide substitutions per site. Lineages under positive selection were indicated with arrow heads.

(D) Maximum-likelihood parameters of selection pressures acting on H. pylori HcpG codons. InL, Log-likelihood score; dN/dS, rate ratio of non-synonymous to synonymous changes averaged over all sites; LRT, likelihood ratio test.

(E) Adaptive evolution in HcpG. Frequency distribution of three codon classes (k1, k2, and k3) and their associated dN/dS ratios were computed under the SSM M3 for HcpG. 11 Codons under positive selection (codon class k3) are shown.
Figure 8: Indels pattern seen among HcpG homologs in diverse H. pylori isolates.

Alignment shown here is generated in Jalview 2.5.1 (ClustalW algorithm) using HcpG homologs from representative populations-Peru (PECAN32), South Africa (R10), Spain (S46, SJM42), Japan (JF58), Korea (K17), India (I44), and European (J99). Deletions of amino acids can be seen as dotted lines throughout the alignment. Alignment is colored based on percentage identity.
Variations in the number and distribution of Sel-1 domains in uninterrupted hcpG ORFs change the tertiary structure of HcpG variants.

Next, I tested if the domain architecture of the encoded proteins was affected as a result of indels in hcpG homologs, using SMART prediction tool. This analysis revealed that indels in uninterrupted ORFs resulted in variations in the number (1 – 7) and distribution of Sel-1 domains in each encoded proteins with a random geographic association in the distribution of HcpG variants (Fig. 7C). Next, I asked if the encoded protein’s tertiary structure was affected due to variations in the number and distribution of Sel-1 domains among diverse HcpG homologs. Comparative protein structure modeling of HcpG variants using the related HcpB and HcpC crystal structures as templates suggested that the tertiary structure of HcpG variants likely differed from each other (Fig. 9). This suggests that the activity or functions of HcpG variants likely differ from each other in subtle or perhaps dramatic manners.

Figure 9: HcpG variants differ in their predicted tertiary structures.
Tertiary structures of HcpG variants from representative populations, varying in the number and distribution of Sel1 domains are depicted here. Numbers in the parenthesis indicate the number of Sel1 domains confidently predicted by SMART analysis. Tertiary structure modeling of HcpG variants was performed using Modeller 9v8; version modweb using the available HcpB and HcpC crystal structures as template.

**hcpG rapidly evolves in diverse *H. pylori* isolates**

To better understand *hcpG* evolutionary dynamics, a maximum-likelihood (ML) phylogeny was reconstructed using the sequence data. Selection pressures on HcpG individual codons and branches of its phylogenetic tree were next studied in detail using two groups of codon-based models of sequence evolution and ML-based LRTs: 1) Site-specific models [SSMs], which examine variation in selection pressures across codons and assume a single $\omega$ across the phylogeny; and 2) Lineage-specific models (LSMs), which allow $\omega$ to vary among lineages, while assuming a single rate across all codons (M1bra). SSMs confidently identified 11 sites under positive selection ($\omega = 3.365$) (Fig 7D & 7E), which suggested different selective pressures at different sites in HcpG. The M1bra model assuming free $\omega$ for each branch was next used to assess the overall variation in $\omega$ in all *hcpG* lineages. The M1bra model also fit the data significantly better than M0 ($p = 0.002$), which suggested that *hcpG* alleles had been subject to different selective pressures in different populations (Fig 7C). Detailed estimates of model parameters are shown in Table 4.
Table 4: Maximum-likelihood parameters of selection pressures acting on *H. pylori* hcpG codons

<table>
<thead>
<tr>
<th>Model Code</th>
<th>InL</th>
<th>Tree-length</th>
<th>$\kappa$</th>
<th>$d_\lambda/d_\alpha$</th>
<th>Parameter Estimates</th>
<th>$D$ [d.f.] $^\dagger$</th>
<th>$\chi^2$</th>
<th>P-value</th>
<th>Positively selected sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>MO (1-ratio)</td>
<td>-524.663</td>
<td>3.2215</td>
<td>4.788</td>
<td>0.325</td>
<td>$\omega = 0.325$</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>M1a (Nearly Neutral)</td>
<td>-5130.388</td>
<td>3.4698</td>
<td>4.867</td>
<td>0.341</td>
<td>$\alpha = 0.742$, $\omega = 0.111$, $p_1 = 0.256$, $\kappa = 1$</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>M2a (Positive Selection)</td>
<td>-5108.067</td>
<td>3.5508</td>
<td>5.228</td>
<td>0.434</td>
<td>$\alpha = 0.723$, $\omega = 0.117$, $p_1 = 0.256$, $\kappa = 1$</td>
<td>M0 vs M2a [3]: 2.291, 4.522, 0.311 M1a vs M2a [2]: 4.462, 0.001</td>
<td>262A</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>M3 (Discrete)</td>
<td>-5105.965</td>
<td>3.551</td>
<td>5.125</td>
<td>0.409</td>
<td>$\alpha = 0.674$, $\omega = 0.039$, $\kappa = 3.365$</td>
<td>M0 vs M3 [4]: 2.739, 0.001 M1a vs M3 [4]: 4.444, 0.001</td>
<td>79L, 221L, 224S, 262A, 313K</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>M7 ($\beta$)</td>
<td>-5135.398</td>
<td>3.4751</td>
<td>4.731</td>
<td>0.321</td>
<td>$\beta = 0.323$, $\kappa = 0.848$</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>M8 ($\beta = 1$)</td>
<td>-5106.761</td>
<td>3.566</td>
<td>5.096</td>
<td>0.405</td>
<td>$\beta = 0.465$, $\kappa = 1.159$, $p_0 = 0.961$, $\kappa = 3.365$</td>
<td>M7 vs M8 [2]: 5.234, 0.0001</td>
<td>210, 262, 305, 365, 58K, 66A, 79L, 221L, 224S, 262A, 313K</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>M8a ($\beta = 0$)</td>
<td>-5128.752</td>
<td>3.525</td>
<td>4.659</td>
<td>0.362</td>
<td>$\beta = 1.516$, $\kappa = 0.983$, $p_0 = 0.733$, $\kappa = 3.365$</td>
<td>M8a vs M8 [1]: 4.364, 0.0001</td>
<td>M1a vs M1bra [3]: 131.1, 0.002</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>M1bra</td>
<td>-5177.115</td>
<td>3.2376</td>
<td>4.649</td>
<td>NA</td>
<td>Free $\alpha$ for each lineage</td>
<td>MO vs M1bra</td>
<td>131.1, 0.002</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

*InL*, Log Likelihood Score  
$^\dagger$, k, kappa, ratio of transition to transversions  
$^\ddagger$, D, Hierarchical Likelihood Ratio Test statistic and d.f., degrees of freedom

Biological relevance of HcpG in *H. pylori* growth and AGS cell infection

Next, I sought to characterize the biological relevance of HcpG in *H. pylori* growth and infection. For this, I first characterized the expression dynamics of HcpG variants *in vitro* and in gastric epithelial cell line (AGS) infection model by qualitative assessment of hcpG transcript using Reverse transcription PCR. These experiments revealed that transcripts of representative HcpG variants were detectable *in vitro* (BHI medium) and in AGS cell infection model (Fig 10A and 10B). Using quantitative real time PCR experiments, I next identified that hcpG transcript is differentially up regulated by 3hr and 6hr post AGS cell infection, in *H. pylori* strains G27MA and J99 (Fig. 10C). Differential regulation of hcpG in different genomic contexts in the AGS infection suggested that hcpG’s role in *H. pylori* strains was likely tightly regulated, which in turn indicated that hcpG is likely biologically relevant during *H. pylori* infection, at least with certain strains. However, previous studies with hcpG (hsp12) had concluded that the
deletion of *hcpG* did not affect *H. pylori* growth [122]. To further characterize the function of HcpG, I generated *hcpG* deletion in *H. pylori* strain G27MA (cell culture adapted strain). Using this strain, I sought to determine the role of HcpG in *H. pylori*’s growth *in vitro* and in AGS cell infection model. My initial findings (Fig. 12B) were in accord with the previous study where it was shown that HcpG did not appear essential for growth of *H. pylori in vitro* [122]. However, since human gastric epithelium constitutes the natural niche for *H. pylori*, I examined the role of HcpG in *H. pylori*’s growth and survival 6 hr and 24 hr post infection in the AGS cell-culture infection model. When compared to the G27MA WT, no significant difference (*p > 0.1*) was found in the growth dynamics of G27MAΔhcpG strain in the AGS cell infection assay (Fig. 12A). These growth dynamics, whereby the growth of parent and the mutant derivative strain was measured in pure culture, provide a measure of absolute fitness. However, such absolute measures of performance can be unreliable in predicting evolutionary success or failure [123]. Competing various strains (here, WT and mutants) in a given environment and monitoring the fitness of each strain relative to the other identifies strains that can perform “better” when both the strains encounter adverse conditions (e.g., nutrient limitation) [123]. Hence, measures of relative fitness should provide more meaningful insights into the contributions of a gene to bacterial growth. In this context, I conducted competition assays between G27MAΔhcpG with G27MAWT *in vitro*, and in AGS cell-culture infection model and determined the relative fitness of G27MAΔhcpG by comparing its growth dynamics with G27MAWT. The results obtained from these experiments indicated that there was no significant difference (*p > 0.1*) in the relative fitness of *H. pylori* strain G27MAΔhcpG when competed with G27MAWT (Fig. 10D).
Given that, *hcpG* was only slightly up regulated (~2 fold) in G27MA compared to J99 (~15 fold), it is reasonable to expect that the effect of *hcpG* deletion will likely be more dramatic in J99 *H. pylori* strain. Thus, attempts at engineering J99 strain to generate *hcpG* mutation however, were unsuccessful (other investigators have had similar difficulties in transforming J99; Dr. Douglas Berg, personal communication). For the purpose of ascertaining that HcpG was expressed during infection with HpG27MA, I conducted infection assays using the G27MA strain wherein the native *hcpG* copy was replaced by an *hcpG::6XHis* insertion (See Materials and Methods section IVD). Even though the *hcpG* transcript was moderately up regulated in G27MA (~2 fold), detectable amounts of HcpG were identified during AGS cell-culture infection (10E), suggesting that the role of HcpG in the context of *H. pylori* strain G27MA cannot be completely eliminated. I monitored the expression of control proteins CagA (translocated into host cells via the CAG-PAI encoded Type IV secretion system [124]), and activated Mitogen-Associated Protein Kinease, MAPK (which is a hallmark of *H. pylori* infection; [125] [126], and tubulin during infection with *H. pylori* G27MA::hcpG-His, to ensure that I replicated known *H. pylori* infection dynamics and to ascertain that equal MOI of *H. pylori* strains was used during this experiment (Fig. 10E). Thus, I conclude that detectable amounts of HcpG are produced by *H. pylori* strain G27MA during infection of AGS cells. However, if HcpG is produced during infection then why didn’t its deletion from G27MA result in any obvious growth defect?
Figure 10: Biological relevance of HcpG during H. pylori growth and infection.

(A) Expression dynamics of hcpG homologs in diverse H. pylori strains grown in vitro. Depicted here is a 1% agarose gel with hcpG homologs amplified by reverse transcription PCR of cDNA synthesized from RNA extracted from indicated H. pylori strains grown in vitro on Brain heart infusion agar plates. recA was amplified as a positive control for H. pylori, and nclf lanes indicate no amplifications from PCR using RNA as a template, suggesting no genomic DNA contamination in cDNA samples.

(B) Expression dynamics of hcpG homologs in diverse H. pylori strains in an AGS cell infection. Depicted here is a 1% agarose gel with hcpG homologs amplified by reverse transcription PCR of cDNA synthesized from RNA extracted from AGS cells infected with indicated H. pylori strains. Infection was proceeded for 6 hrs before RNA was extracted. recA and gapdh were amplified as a positive controls for H. pylori and AGS cells respectively, and nclf lanes indicate no amplifications from PCR using RNA as a template, suggesting no genomic DNA contamination in cDNA samples.

(C) Differential expression of hcpG in H. pylori strains, J99 and G27MA in an AGS cell infection. 3 hr and 6 hr post AGS infection, strain J99 shows a striking up regulation of hcpG transcript by 25 fold and 16 fold, respectively. Compared to J99, strain G27MA shows a mild up regulation (~2 fold) of its hcpG transcript, 3 hr and 6 hr post infection.

(D) Relative fitness measures of G27MAΔhcpG. No significant reduction in the relative fitness of H. pylori strain G27MAΔhcpG was observed when competed with G27MA WT in an AGS cell infection for 6 hr and 24 hr. Graph in the left
panel depicts colony forming units (CFUs) obtained for each WT and mutant strain when plated on antibiotic resistant BHI agar plates after competing for indicated time points. Gel image in the inset shows fragment ABC amplified from G27MA WT (strR) and G27MAΔhcpG (catR) H. pylori colonies. hcpG mutant ABC fragment is 600bp heavier than WT ABC fragment due to insertion of rpsl- cat cassette at hcpG locus. Error bars represent standard deviations. Panel to the right depicts box plot representation of competitive indices calculated from the CFUs in the left panel. Bar located in the box represents the median value. CI > 1 indicates that mutant is favored over the WT and CI < 1 indicates that WT is favored over the mutant. Values of CI at 6 hr = 1.08, 24 hr = 0.92 indicated that there is no significant reduction in the relative fitness of G27MAΔhcpG (p > 0.1) in competition with G27MA WT.

(E) Detection of G27MA HcpG in an AGS cell infection. Depicted here is an immuno blot detecting G27MA HcpG-His in an AGS cell infection. Lane 1 has cell lysates from uninfected serum starved AGS cells. Lanes 2 and 5 have cell lysates from AGS cells infected with G27MA-hcpG:His at 3hr and 6 hr respectively. Lanes 3 and 5 have cell lysates from AGS cells infected with G27MA-hcpGΔ at 3hr and 6 hr respectively. α- HcpG-His antibody detects HcpG-His. CagA blot serves as a control for equal MOIs of G27MA-hcpG:His and G27MA-hcpGΔ in AGS cell infection. MAPK-YT blot serves as a control for H. pylori infection dynamics in AGS cells (activated MAPK is a hallmark of H. pylori infection), and Tubulin blot serves as a loading control.

Non-neutral evolutionary dynamics of hcpC

Phylogenetic analysis of slr gene homologs from closely related ε-proteobacteria revealed a H. pylori genome specific slr gene family expansion by gene duplication, driven by positive selection, possibly for functional diversity [42]. I hypothesized that one reason for the lack of any obvious contribution by HcpG to bacterial growth was the likely presence of its paralog, HcpC, in H. pylori genome. Thus, as a first step towards elucidating any dynamic interplay between HcpC and HcpG during H. pylori infection, I sought to elucidate the evolutionary dynamics of the closest homolog or ancestor of hcpG in H. pylori genomes. G27MA hcpG is 61.4% (DNA) and 44.5% (amino acid) identical to G27MA hcpC homolog (Fig. 11A & 11B). To better understand hcpC evolutionary dynamics, complete nucleotide sequences from European (Spain), East Asian (Japan and
Korea), South American (Peru, Shima) and African (Gambia) \textit{H. pylori} strains (N=100) were determined. The ML phylogeny reconstructed using the sequence data of a subset of \textit{hcpC} alleles (N=81) revealed strong geographic clustering among \textit{H. pylori} \textit{hcpC} sequences, which was strikingly absent among \textit{hcpG} alleles. (Fig. 11C). Such strong subdivision is very typically seen in most \textit{H. pylori} genes. There were, however, several examples wherein alleles of one geographic origin clustered with those from another, which suggested a history of recent admixture. Selection pressures on HcpC individual codons and branches of its phylogenetic tree were next studied in detail. SSMs confidently identified 23 sites under positive selection (\(\omega = 1.4\)) (Fig. 11D & 11E), which suggested different selective pressures at different sites in HcpC. The M1bra model was next used to assess the overall variation in \(\omega\) in all \textit{hcpC} lineages. This model also fit the data significantly better than M0 (p = 0.003) (Fig. 11D), which suggested that \textit{hcpC} alleles had been subject to different selective pressures in different populations (Fig. 11C). Detailed estimates of ML parameters associated with each model are shown in Table 5.
Figure 11: Non neutral evolutionary dynamics of hcpC.

(A) G27MA HcpG is 44.5% identical to G27MA HcpC. Depicted here is a pairwise clustalW alignment generated in Jalview 2.5.1 using HcpC (G27_1039) and HcpG (G27_1469) homologs in G27MA. Blue shaded regions show similarity between the two protein sequences.

(B) Depicts HcpG amino acid residues identical with HcpC, mapped onto HcpC crystal structure.
The phylogeny was reconstructed assuming the TrN +I+G substitution model and was optimized to the following parameters using heuristic searches and tree bisection-reconnection algorithm: rate matrix: \( A \rho C = 1, A \rho G = 6.5621, A \rho T = 1, C \rho G = 1, C \rho T = 11.8894, \) and \( G \rho T = 1; \) base frequencies: \( A = 0.31, C = 0.15, G = 0.27, T = 0.25 \) proportion of invariable sites, \( I = 0.5367 \) and \( \Gamma \) distribution shape parameter = 0.3544. Significant bootstrap values (≥50) are shown. Phylogeny is rooted using the out group method implemented in PAUP. Bar scale = 0.01 nucleotide substitutions per site. Lineages under positive selection were indicated with arrow heads.

**Table 5: Maximum-likelihood parameters of selection pressures acting on *H. pylori* hcpC codons**

<table>
<thead>
<tr>
<th>Model Code</th>
<th>InL *</th>
<th>Tree-length</th>
<th>( \chi^2 )</th>
<th>( d_N/d_S )</th>
<th>Parameter Estimates</th>
<th>D [d.f.]</th>
<th>( \chi^2 )</th>
<th>P-value</th>
<th>Positively selected sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>MO (1-ratio)</td>
<td>-6431.304</td>
<td>3.358</td>
<td>3.448</td>
<td>0.1311</td>
<td>( \alpha_0 = 0.1311 )</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>M1a (Nearly Neutral)</td>
<td>-6137.837</td>
<td>3.447</td>
<td>3.478</td>
<td>0.135</td>
<td>( \theta_{1} = 0.881, \theta_0 = 0.018; \theta_{1} = 0.119, \theta_0 = 1 )</td>
<td>M0 vs M2a [3]</td>
<td>19.832</td>
<td>&lt;0.0001</td>
<td>34, 43Q, 75Q, 161T, 229M, 248V</td>
</tr>
<tr>
<td>M2a (Positive Selection)</td>
<td>-6127.888</td>
<td>3.499</td>
<td>3.82</td>
<td>0.171</td>
<td>( \theta_0 = 0.878, \theta_0 = 0.018; \theta_{1} = 0.102, \theta_0 = 1 )</td>
<td>M0 vs M2a [2]</td>
<td>19.898</td>
<td>&lt;0.0001</td>
<td>34, 43Q, 75Q, 161T, 229M, 248V</td>
</tr>
<tr>
<td>M3 (Discrete)</td>
<td>-6124.796</td>
<td>3.483</td>
<td>3.665</td>
<td>0.155</td>
<td>( \theta_0 = 0.794, \theta_0 = 0.004; \theta_{1} = 0.119, \theta_0 = 1 )</td>
<td>M0 vs M3 [4]</td>
<td>21.208</td>
<td>&lt;0.0001</td>
<td>179A, 192G, 210D, 212V, 213A, 229M, 230Q, 248V, 249T, 273L</td>
</tr>
<tr>
<td>M7 (( \beta ))</td>
<td>-6131.577</td>
<td>3.389</td>
<td>3.452</td>
<td>0.117</td>
<td>( \theta_0 = 0.021, \theta_0 = 0.145 )</td>
<td>M0 vs M8 [2]</td>
<td>19.898</td>
<td>&lt;0.0001</td>
<td>34, 43Q, 75Q, 161T, 229M, 248V</td>
</tr>
<tr>
<td>M8 (( \beta, \phi &gt; 1 ))</td>
<td>-6126.782</td>
<td>3.547</td>
<td>3.742</td>
<td>0.162</td>
<td>( \theta_0 = 0.045, \theta_0 = 0.375; \theta_{1} = 0.978 )</td>
<td>M0 vs M8 [2]</td>
<td>21.41</td>
<td>&lt;0.0001</td>
<td>86G, 89A, 95Q, 161T, 192G, 210D, 212V, 213A, 229M, 232Q, 248V, 249T, 273L</td>
</tr>
<tr>
<td>M1a (( \beta, \phi &lt; 1 ))</td>
<td>-6129.478</td>
<td>3.464</td>
<td>3.426</td>
<td>0.127</td>
<td>( \theta_0 = 0.102, \theta_0 = 0.291, \theta_{1} = 0.056 )</td>
<td>M8a vs M8 [1]</td>
<td>17.12</td>
<td>&lt;0.0001</td>
<td>NA</td>
</tr>
<tr>
<td>M1bra</td>
<td>-6334.72</td>
<td>3.362</td>
<td>3.458</td>
<td>NA</td>
<td>Free ( \alpha ) for each lineage</td>
<td>M0 vs M1bra</td>
<td>213.168</td>
<td>&lt;0.004</td>
<td>NA</td>
</tr>
</tbody>
</table>

* InL, Log-likelihood Score
\( \Gamma \), kappa, ratio of transition to transversions
\( \beta \), D, hierarchical Likelihood Ratio Test statistic and d.f., degrees of freedom
Biological significance of HcpC adaptive evolution

To examine adaptive evolution in HcpC in a protein-structure function context, amino acids identified to be under positive selection were mapped on the crystal structure of HcpC in collaboration with Dr. Peer Mittl at the University of Zurich. Most adaptive amino acids mapped to the molecular surface of the HcpC protein (Fig. 11F), which was reminiscent of our findings with other H. pylori sir genes [42] and suggested that positive selection may affect the strength or specificity of interaction. Taken together, the presence of hcpC in all H. pylori strains tested, and evidence of positive selection superimposed upon strong geographic clustering suggest a likely essential role of hcpC in H. pylori infection.

Growth kinetics of G27MAΔhcpC and G27MAΔhcpCΔhcpG

To further characterize the functional dynamics of HcpC, I assessed the role of HcpC in H. pylori's survival and reproduction in vitro and in AGS cell-culture infection model. For this purpose, I conducted growth assays with hcpC single mutant, and a double mutant lacking both hcpC and hcpG in H. pylori strain G27MA. No significant defect (p > 0.1) was observed in the growth rate of G27MAΔhcpC 6 hr (lag phase), 24 hr (mid exponential phase), 48 hr (stationary phase), and 56 hr (death phase) grown in vitro. However, the G27MAΔhcpCΔhcpG strain exhibited a significant growth defect (0.01 > p < 0.05) 48 hr and 56 hr when grown in vitro (Fig. 12B). This finding suggests that nutrient scarcity poses adverse effects to the absolute fitness of double mutant G27MAΔhcpCΔhcpG grown in vitro. However, no significant reduction (p > 0.1) was observed in the relative fitness of both G27MAΔhcpC (Fig. 12D) and G27MAΔhcpCΔhcpG mutant strains (Fig. 12F), when competed with G27MA WT in
vitro. Next, I examined the role of HcpC and HcpG in *H. pylori*’s growth and survival 6 hr and 24 hr post AGS cell infection. When compared to the G27MA WT, significant difference (0.01 > *p* < 0.05) was found in the growth dynamics of *G27MAΔhcpC* and *G27MAΔhcpCΔhcpG* (Fig. 12A) strains 24 hr post infection. This finding suggested a possible role of HcpC relatively late infection in the AGS cell culture model.

**HcpC and HcpG paralogs are redundant and contribute additively to relative fitness of *H. pylori* strain G27MA in AGS cell infection**

Next, I assessed for relative fitness defects (if any) in *G27MAΔhcpC* and *G27MAΔhcpCΔhcpG* mutants in AGS cell infection model. Results showed that both the tested mutants showed a significant (*p* < 0.01) reduction (30% decreased relative fitness in *hcpC* mutant and 40% in *hcpC*, *hcpG* double mutant) in the relative fitness of respective bacterial strains at 24 hr post infection, but only the double mutant showed a significant reduction (*p* < 0.01) (25% reduced relative fitness), 6 hrs post infection (*p* < 0.01) (Fig. 12C & 12E). Thus my results from 6 hrs post infection show that while loss of either *hcpC* or *hcpG* alone does not adversely affect *H. pylori* fitness, lack of both *hcpC* and *hcpG* significantly affected the ability of *H. pylori* to grow during infection, thereby revealing that *hcpG* and *hcpC* are genetically redundant. Similarly, while *hcpG* deletion had no significant impact on *H. pylori* growth, lack of *hcpC* significantly reduced *H. pylori*’s ability to compete with the WT parent strain at 24 hrs post infection. Strikingly, the mutant derivative lacking both *hcpC* and *hcpG* performed worse than the WT or *hcpG* and *hcpC* mutants themselves. This outcome suggested that HcpC and HcpG were not only genetically redundant, their contribution to *H. pylori* fitness was additive.
Figure 12: Growth and relative fitness dynamics of G27MA – hcp mutants.

(A) Growth kinetics of G27MA – hcp mutants in an AGS cell infection. The infection was carried out in triplicate using the indicated G27MA mutants and G27MA WT with a MOI of 100. G27MAΔhcpC and G27MAΔhcpCΔhcpG mutant strains exhibited a significant (0.01 > p < 0.05) decrease in the growth rate 24 hr post infection when compared with G27MA WT. Error bars indicate standard deviations.

(B) Growth kinetics of G27MA – hcp mutants grown in vitro.
With a starting bacterial density OD$_{600}$ of 0.05 / ml, BHI broths were inoculated with the indicated G27MA – hcp mutants and G27MA WT and grown for indicated time points. Only the G27MAΔhcpCΔhcpG double mutant exhibited a significant growth defect (0.01 > p < 0.05) when grown for 48 hr and 56 hr in vitro. Error bars represent standard deviations from four independent experiments.

(C) Relative fitness measures of G27MAΔhcpC in AGS cell infection. Significant reduction in the relative fitness of H. pylori strain G27MAΔhcpC was observed when competed with G27MA WT in an AGS cell infection for 24 hr. Graph to the left depicts colony forming units (CFUs) obtained for each WT and mutant strain when plated on antibiotic resistant BHI agar plates after competing for indicated time points. Gel image in the inset shows fragment ABC amplified from G27MA WT (str$^R$) and G27MAΔhcpC (erm$^R$) H. pylori colonies. hcpC mutant ABC fragment is 1400bp larger than WT ABC fragment due to insertion of rpsl-erm cassette at hcpC locus. Error bars represent standard deviations of four independent experiments. Graph to the right depicts box plot representation of competitive indices calculated from the CFUs in the left panel. Bar located in the box represents the median value. CI > 1 indicates that mutant is favored over the WT and CI < 1 indicates that WT is favored over the mutant. Values of CI at 6 hr = 0.97, 24 hr = 0.73 indicated that there is a 27% reduction in the relative fitness of G27MAΔhcpC (p < 0.001) in competition with G27MA WT.

(D) Relative fitness measures of G27MAΔhcpC grown in vitro. Graph depicted here is a box plot representation of competitive index (CI). Values of CI at 6 hr = 0.92, 24 hr = 0.98 indicated that there is no significant reduction in the relative fitness of H. pylori strain G27MAΔhcpC when competed with G27MA WT in vitro, and grown for 12 hr and 56 hr.

(E) Relative fitness measures of G27MAΔhcpCΔhcpG in AGS cell infection. Significant reduction (p < 0.001) in the relative fitness of H. pylori strain G27MAΔhcpCΔhcpG was observed when competed with G27MA WT in an AGS cell infection for 6hr and 24 hr. Graph to the left depicts colony forming units (CFUs) obtained for each WT and mutant strain when plated on antibiotic resistant BHI agar plates after competing for indicated time points. Gel image in the inset shows fragment ABC amplified from G27MA WT (str$^R$), G27MAΔhcpC (erm$^R$), and G27MAΔhcpG (cat$^R$) H. pylori colonies. Graph to the right depicts box plot representation of competitive indices calculated from the CFUs in the left panel. Bar located in the box represents the median value. Values of CI at 6 hr = 0.74, 24 hr = 0.56 indicated that there is a 27% reduction in the relative fitness of G27MAΔhcpCΔhcpG (p < 0.001) in competition with G27MA WT.

(D) Relative fitness measures of G27MAΔhcpCΔhcpG grown in vitro. Graph depicted here is a box plot representation of competitive index (CI). Values of CI at 6 hr = 0.94, 24 hr = 1.09 indicated that there is no significant (p > 0.1) reduction in the relative fitness of H. pylori strain G27MAΔhcpCΔhcpG when competed with G27MA WT in vitro, and grown for 12 hr and 56 hr.
Role of HcpC and HcpG in surface translocation of HspB

Even though the SEL1-like domains are often generally involved in protein-protein interactions, [43, 55, 127-130] little is known about the biological function of Helicobacter SLR Hcp proteins. The crystal structure of HcpC had suggested a peptide binding site in its crystal contact I surface, which was similar to the binding site seen in eukaryotic TPR protein Hsp70/Hsp90 organizing protein (Hop) [131]. H. pylori genomes encode 10 - 11 heat shock proteins; among these the 58 kDa heat shock protein B (HspB or GroEL) is unique in that, unlike other bacteria where it is cytoplasmic, in H. pylori HspB is also found on the bacterial surface in association with Urease subunit UreB [132-135]. Given these findings, and that eukaryotic Sel-1 proteins are known to function as chaperones in assembling macro molecular complexes [49-52], I hypothesized that hcpC/hcpG might function as chaperone proteins, likely involved in translocation of HspB to H. pylori membrane. Thus, HspB surface expression in AGS infection assays with G27MA WT or G27MAΔhcpC or G27MAΔhcpG or G27MAΔhcpCΔhcpG was quantified using a BD FACS Calibur (Beckton Dickenson Inc., USA) at 3 hr, 6 hr, 12 hr and 24 hr post AGS infection. This analysis clearly indicated that all the G27MA - hcp mutants tested showed a significant ($p < 0.01$) defect in the surface localization of HspB compared to the WT G27MA strain at 3 hr post infection (Fig. 13A). However, only the strains lacking hcpC and hcpC & hcpG showed a significant defect ($p < 0.01$) in surface localization of HspB at 6 hr (Fig. 13B), 12 hr (Fig. 13C) and 24 hr (Fig. 13D) post infection. These findings suggest that hcpC and hcpG can each modulate HspB surface expression, and that HcpC can compensate for the loss of HcpG in G27MAΔhcpG strain by 6hr post infection. However, HcpG is unable to compensate for the lack of HcpC as
illustrated by HspB expression dynamics during infection with G27MAΔhcpC infection, whereby the hcpC mutant showed significant reduction in HspB surface expression throughout the infection time course (Fig. 13). Significantly, the HspB dynamics during infection with G27MAΔhcpCΔhcpG and G27MAΔhcpC were quite similar. These findings suggest the following conclusions: 1) hcpC is necessary and sufficient for optimal surface expression of HspB; 2) that hcpC and hcpG are functionally redundant but in a non-reciprocal fashion; and 3) that non-reciprocal functional redundancy stems from the relatively higher efficiency of hcpC to regulate HspB surface expression. Dynamics of CagA and phosphorylated MAPK were monitored as controls to ensure the specificity of HspB dynamics in G27MA - hcp mutants (Fig. 14). These data showed that parameters of H. pylori infection, independent of HcpG and HcpC, remained unaffected, thereby supporting my observation that HcpC and HcpG function to specifically modulate surface exposure of HspB antigen.
Figure 13: Role of G27MA HcpC and G27MA HcpG in *H. pylori* surface translocation of HspB.
(A) Defect in surface localization of Hsp8 in all the tested G27MA – hcp mutants, 3 hr post AGS infection.

Depicted here is a histogram representation of mean fluorescence of surface expressed Hsp8; black – G27MA WT, blue – G27MAΔhcpC, red – G27MAΔhcpG, green – G27MAΔhcpCΔhcpG. x – axis indicates Hsp8 fluorescence; y- axis indicates number of events. Decrease in the amount of fluorescence shifts the peaks to left (here, blue, red and green peaks compared to the black peak). Graph in the inset is plotted using geometric mean intensity of Hsp8 fluorescence to calculate statistical significance. Significant difference (p < 0.01) in geometric mean intensity of Hsp8 fluorescence was detected in all the G27MA – hcp mutants compared to G27MA WT. Error bars represent standard deviations from three independent experiments.

(B) Defect in surface localization of Hsp8 in G27MAΔhcpC, and G27MAΔhcpCΔhcpG, 3 hr, 6hr (C), and 24 hr (D) post AGS infection.

Following 6 hr post AGS infection, G27MAΔhcpG strain did not exhibited a defect in surface translocation of Hsp8, whereas G27MAΔhcpC, and G27MAΔhcpCΔhcpG strains consistently exhibited the defect. Error bars represent standard deviations from three independent experiments. * indicates statistical significance.
Figure 14: Dynamics of CagA and MAPKYT in G27MA – AGS infection.
Depicted here is a box plot representation of CagA florescence (x-axis) and MAPKYT fluorescence (y-axis) of the AGS cells infected with indicated G27MA mutants and G27MA WT strain. As expected, in uninfected AGS control, the CagA (control for equal MOI) and MAPKYT (activation is a hallmark of host epithelial cells following H. pylori infection) fluorescence is very low, which were then up regulated post AGS infection with indicated H. pylori strains. Following AGS infection for indicated time points, no difference was observed in CagA, and MAPKYT dynamics among G27MA WT and G27MA - hcp mutants. This suggests that dynamics seen in HspB surface localization was specific to G27MA - hcp mutants.

Next, I asked if the variation in HspB surface localization in G27MA - hcp mutants was indeed due to a defect in surface translocation of HspB or, as a result of decreased synthesis of HspB in the G27MA - hcp mutant bacterial strains. To test this, I assessed the expression dynamics of hspB in the G27MAWT and G27MA - hcp mutant bacterial strains in the AGS cell infection model using quantitative real time PCR. Since, H. pylori HspB is closely associated with Urease [136], I also tested the expression dynamics of urease functional sub unit, ureB in G27MA - hcp mutants, post AGS cell infection. Results from these experiments indicated that there was no apparent reduction i.e., <2 fold in the amount of hspB (Fig. 15B), and ureB (Fig 15C) transcript compared to the WT strain, 3 hr and 6 hr post AGS infection. Taken together, I conclude that only surface translocation of HspB is affected in the tested G27MA - hcp mutants and that both HcpC and HcpG play a role in transporting HspB from cell cytosol to the bacterial surface, 3 hr post infection. However, by 6 hr post infection, HcpC compensates for the loss of HcpG in G27MAΔhcpG strain as no defect in HspB surface localization was observed in G27MAΔhcpG strain from this time interval. A consistent defect was shown by the hcpC mutant however, indicating a non-reciprocal effect of HcpG on loss of hcpC in H. pylori strain G27MAΔhcpC.
Next, I sought to characterize the compensatory mechanism of G27MA HcpC in G27MAΔhcpG mutant strain. I hypothesized that G27MA HcpC might have played a role in rescuing the defect of HspB surface translocation in G27MAΔhcpG via its transcript up regulation by 6 hrs post infection. To test this hypothesis, I quantified the level of hcpC transcript in G27MA WT *H. pylori* strain and G27MAΔhcpG strain, 3 hr, 6 hr, and 24 hr post AGS infection. However, results from this analysis indicated no apparent up regulation i.e., > 2 fold of hcpC transcript in G27MAΔhcpG mutant strain (Fig. 15A). Yet, it is still possible that, other regulatory mechanisms or stability of HcpC might have played a role in rescuing the observed defect.

**Figure 15: Synthesis of HspB is not affected in G27MA – hcp mutants.**

(A) Regulation of G27MA - hcpC transcript expression in G27MA - hcpG mutant. qRT-PCR was used to determine the regulation of expression of hcpC and hcpG in G27MA WT and in indicated G27MA - hcp mutants. There is no apparent up regulation of either hcpC or hcpG transcripts in the absence of their paralogs. The results are represented as relative quantification normalized to *ureA*. Error bars represent standard deviations of triplicate experiments.

(B) Expression dynamics of hspB in G27MA - hcp mutants. Real Time qRT-PCR was used to determine the regulation of expression of hspB in G27MA WT and indicated G27MA - hcp mutants, 3hr and 6hr post AGS infection. There is no apparent down regulation of HspB transcript in G27MA – hcp mutants' following AGS infection, suggesting that only surface translocation of HspB was affected. The results are represented as relative quantification normalized to *ureA*. Error bars represent standard deviations of triplicate experiments.
quantification normalized to recA. Error bars represent standard deviations of triplicate experiments.

(C) Expression dynamics of ureB in G27MA - hcp mutants.

Real Time qRT-PCR was used to determine the regulation of expression of ureB in G27MA WT and indicated G27MA - hcp mutants, 3hr and 6hr post AGS infection. There is no apparent up / down regulation of UreB transcript in G27MA – hcp mutants' following AGS infection. The results are represented as relative quantification normalized to recA. Error bars represent standard deviations of triplicate experiments.

HcpC and HcpG dependent modulation of HspB surface expression requires cellular infection.

Next, I asked if the defect seen in HspB surface localization in G27MA – hcp mutants is specific to the presence of host cellular components (modeled by AGS infection) or a constitutive property of G27MA - hcp mutants in vitro (BHI liquid culture). To determine this, I measured and compared the fluorescence of bacterial surface associated HspB in G27MA WT and G27MA – hcp mutants that are grown for 3 hr, 24 hr, and 56 hr in vitro. Comparison of mean geometric fluorescent intensities of the WT and hcp mutants revealed no difference in the mean fluorescence among the strains tested grown in vitro for 3 hr, 24 hr, and 56 hr (Fig. 16A, 16B, and 16C, respectively). The 56 hr time point was included as G27MAΔhcpCΔhcpG double mutant grown for 56 hr in vitro, exhibited a significant defect (0.01 > p < 0.05) in its growth rate (Fig. 12B). However, as shown above, decreased growth rate had no effect in HspB surface translocation. Hence, although the number of G27MAΔhcpCΔhcpG were significantly less than the G27MA WT by 56 hrs when grown in vitro, the amount of HspB translocated to the bacterial surface was not altered in the double mutant. Taken together,
my data indicate that the defect in HspB surface localization exhibited by G27MA – hcp mutants is specific to the cellular infection model.

Figure 16: Cellular infection dependent surface translocation defect of HspB in G27MA – hcp mutants.

(A) HspB surface localization in G27MA - hcp mutants in vitro.
No defect is observed in the surface localization of HspB in G27MA - hcp mutants grown in vitro for 3hr; (B) 24hr; and (C) 56hr. HspB surface expression was detected by measuring fluorescence of HspB expressed on the surface of bacteria, using a BD FACS Calibur machine.

Summary I:

1. \textit{hcpG} is only present in a subset of \textit{H. pylori} strains, and in a significant number of \textit{H. pylori} strains \textit{hcpG} is pseudogenized or deleted.
2. Striking sequence variability was found in \textit{hcpG} homologs in diverse \textit{H. pylori} isolates.
3. \textit{hcpC} and \textit{hcpG} evolve rapidly under positive selection and HcpC contributes significantly to \textit{H. pylori} growth and fitness during infection.
4. HcpC and HcpG are genetically redundant and contribute additively to \textit{H. pylori} fitness.
5. HcpC is necessary and sufficient for optimal surface expression of HspB.
6. Absence of HcpG downregulates HspB surface expression during very early infection and its loss is compensated for by HcpC thereby implying that HcpC and HcpG are functionally redundant.

7. HcpG alone does not rescue the lack of HcpC, suggesting that the observed functional redundancy is non-reciprocal.

II. Role of HepC during *H. pylori* Growth and Infection.

**Differential regulation of hepC expression in diverse *H. pylori* isolates in AGS cell infection model**

Earlier studies indicated a likely role of HepC in the adaptation of *H. pylori* to diverse human hosts [42]. Thus, to elucidate the biological relevance of HepC in *H. pylori* infection, I first monitored the expression dynamics of *H. pylori* strain G27MA hepC grown *in vitro* and in AGS cell infection model by Reverse transcription PCR. These experiments revealed that HepC transcript was detectable *in vitro* (BHI medium) and in the AGS cell infection model (Fig. 17A). Since hepC evolves rapidly in diverse *H. pylori* populations with different amino acids often being selected in different geographic regions likely fine tuning host responses [42], I hypothesized that genetically diverse *H. pylori* isolates differentially regulate hepC transcript expression. Therefore, I used quantitative real time PCR to obtain a quantitative measure of the hepC transcript in AGS cell infection of diverse *H. pylori* strains. As expected, this analysis revealed that genetically diverse *H. pylori* strains differentially regulate expression of hepC transcript (Fig. 17B). More importantly, I determined that the Japanese *H. pylori* strain, JS7 exhibited a maximal up regulation in hepC expression (~ 10 fold), 3 hr and 6 hr post infection compared to *H. pylori* strains isolated from European (26695), Amerindian
(Shi470) and South African (R10) populations (Fig. 17B). Such differential regulation of \( hepC \) in different genomic contexts in the AGS infection suggested that \( hepC \)'s role in \( H. pylori \) strains was likely tightly regulated, which in turn indicated that HepC is likely biologically relevant during \( H. pylori \) infection.

**HepC contributes significantly to the fitness of \( H. pylori \)**

To further characterize the biological significance of HepC in \( H. pylori \) infection, I then sought to determine the contribution of HepC to \( H. pylori \)'s fitness in an AGS cell infection model. Since relative fitness measures provide more accurate measures at understanding evolutionary success \([121]\), I determined the relative fitness of \( G_{27MA\Delta hepC} \) strain compared to \( G_{27MA \text{ WT}} \) \( H. pylori \) strain for survival and replication, in AGS cell infection model. Results from competition assay revealed that \( G_{27MA\Delta hepC} \) mutant showed a significant reduction (30% reduction) in the relative fitness, 24 hr post infection (Fig. 17D) (student's \( t \)-test; \( p < 0.01 \)) and a non-significant reduction 6 hr post infection (Fig. 17D). Since, \( G_{27MA\Delta hepC} \) exhibited a significant reduction in relative fitness compared to \( G_{27MA \text{ WT}} \), 24 hr post infection but not at 6 hr, I hypothesized that HepC plays a crucial role late in the infection. To test this hypothesis, I monitored the expression pattern of \( hepC \) in \( G_{27MA} \) - AGS infection to detect if \( hepC \) expression was significantly up regulated 24 hrs post infection. As expected, there was an apparent up regulation (~13-fold) of \( hepC \) transcript 24 hr post infection when compared to its expression pattern at 3 hr and 6 hr post AGS infection (Fig. 17B).
Figure 17: hepC appears to be relevant biologically, and required during H. pylori infection.

(A) hepC mRNA is detected during in vitro growth and 6 hours following infection of AGS cells. Depicted here is a 1% agarose gel with G27MA hcpC homolog amplified by reverse transcription PCR of cDNA synthesized from RNA extracted from G27MA grown in vitro on Brain heart infusion agar plates and 6 hr post AGS cell infection. recA and gapdh were amplified as positive controls for H. pylori and AGS cell infection, respectively. nct lanes indicate no amplifications from PCR using RNA as a template, suggesting no genomic DNA contamination in cDNA samples.

(B) Real-time PCR analysis shows that the hepC transcript is dramatically upregulated (~13-fold) following 24 hrs of infection with H. pylori strain G27MA.

(C) (D) Deletion of hepC reduces the ability of G27MA△hepC to grow and compete with the WT resulting in a 30% reduction in the relative fitness of G27MA△hepC, 24 hours post infection.

(E) Real-time PCR analysis shows that hepC is differentially regulated in different genomic contexts.
HepC likely targets the host cytoskeletal machinery during late infection in AGS cell culture model of infection

Ongoing work from other investigators in the lab demonstrated that HepC interacts with the multifunctional human cytoskeletal proteins Ezrin, and Vinculin. Therefore, I sought to determine the aspect of host cytoskeleton that HepC targets. Cell scattering or humming bird phenotype of AGS cells is a result of *H. pylori* infection mediated cytoskeletal dysregulation, and is a hallmark of *H. pylori* infected AGS cells [83, 84]. With this rationale and given that HepC interacts with human cytoskeletal proteins, Ezrin and Vinculin, I first asked if HepC has a role in cell scattering of AGS cells. To test this, I infected AGS cells with *H. pylori* strains G27MA WT, G27MAΔhepC, G27MAΔvirD4 (a type IV secretion system deletion mutant which is unable to induce cell scattering phenotype), 26695 WT, 26695ΔhepC, and measured the cell scattering phenotype 6 hr post infection. I identified that both the *hepC* mutants tested mediated cell scattering phenotype similar to their respective WT strains (Fig. 18A). This finding suggested that HepC is not likely to mediate early cytoskeletal changes. However, as our earlier finding (Fig. 17C, 17D & 17E) indicated a likely role of HepC, late in the infection, I then sought to determine the molecular basis of cytoskeletal deregulation (if any) by HepC, 24 hr post AGS infection. Quantifying the cell scattering phenotype 24 hrs post infection posed a challenge due to extreme mobility of cells. Using cytoskeletal pathway specific PCR arrays I quantified the relative expression dynamics of cytoskeletal regulators in the G27MAΔhepC mutant compared to the G27MA WT, 24 hrs post infection, to identify cytoskeletal regulators that were specifically targeted by HepC. As expected, scatter plot analyses demonstrating > or < 2 fold differences in gene
expression, identified 10 genes were identified that were apparently up regulated and 1 gene that was apparently down regulated in G27MAΔhepC infection when compared to G27MA WT infection (Fig. 18B). Inferences from this experiment are as follows: 1) cytoskeletal regulators that were identified as up regulated in G27MAΔhepC infection were likely down regulated by HepC in WT infection; and that 2) cytoskeletal regulators that were identified as down regulated in G27MAΔhepC infection were likely up regulated by HepC in WT infection. Such dysregulation of cytoskeletal regulators by HepC, 24 hrs post infection suggests that HepC most likely targets host cytoskeletal machinery during late infection in a cell culture model of infection.
Figure 18: HepC likely targets the host cytoskeletal machinery during late infection in AGS cell culture model of infection.

(A) HepC does not mediate early cytoskeletal changes following infection. AGS cells were infected at an MOI of 100 with indicated bacterial strains. virD4 mutant is a Type IV secretion system mutant and is used as a control for cell scattering. 6hr post infection, cell extensions measuring over 40 μmeters were considered “scattered”. No difference was observed in the scattering phenotype of AGS cells infected with WT or hepC mutant H. pylori strains. Experiment was done in triplicate and error bars represent standard deviations.

(B) HepC likely targets host cell cytoskeleton during later (24 hrs) period of AGS cell infection. Scatter plot analysis comparing expression of cytoskeletal regulators following 24hrs post infection with Log10 2^ΔCt values of G27MA hepC mutant on y-axis and Log10 2^ΔCt values of G27MA WT on x-axis. Number of genes significantly up (≥ 2 fold) and down regulated (< 2 fold)
are shown in red and green circles, respectively. 10 genes were likely down regulated by HepC, 24 hr post AGS cell infection.

**Genetically diverse *H. pylori* strains differentially dysregulate cytoskeletal regulators during an early infection of AGS cell line infection**

Other investigators in our lab demonstrated that different geographic variants of HepC differ in their binding affinity to Ezrin. Given that Ezrin is a major cytoskeletal regulator that influences diverse cytoskeleton-dependent cell functions, that geographically distinct *H. pylori* strains interact differently with Ezrin, and that cytoskeletal dysregulation is a hallmark of *H. pylori* infection [83, 84], I then hypothesized that geographically distinct *H. pylori* strains likely differ in their ability to deregulate host cytoskeletal dynamics. To test this hypothesis, I first assessed for the humming bird phenotype (a consequence of cytoskeletal deregulation by *H. pylori*) of AGS cells infected with genetically diverse *H. pylori* strains. For this, I infected AGS cells with diverse *H. pylori* strains isolated from patients belonging to specific human populations: Amerindian (*Shi470*), Japan (*JS7*), Europe (*J99*), and cell culture adapted strain (*G27MA*), and monitored cell scattering phenotype, 6 hr post infection. Results from this experiment showed that *H. pylori* isolates from different geographic regions can differentially affect the cell scattering phenotype in AGS cells (*p* value < 0.05) (Fig. 19A & 19B). More importantly, Japanese *H. pylori* strain JS7 induced cell scattering at a higher rate as compared to infection with other tested strains. This finding is interesting in that cell scattering is a result of loss of cell-cell junctions, cytoskeletal modifications and eventual acquisition of motility, which are important in cancer progression and metastasis [84 - 86] and *H. pylori* mediated gastric cancer is most common in Japanese
populations [33, 34]. Next, I sought to further characterize and quantify the molecular basis of cytoskeletal deregulation by diverse *H. pylori* strains. To determine this, the expression of cytoskeletal regulators in AGS cells infected with diverse *H. pylori* strains for 3 hr and 6 hr, was compared with the expression of cytoskeletal regulators in uninfected AGS cells using cytoskeletal pathway specific PCR arrays, to identify genes that were differentially up or down regulated following infection. Analysis of the results obtained from this experiment clearly demonstrated the following key features (Fig. 19C):

1. Different genes were differentially up- or down-regulated at any given time point.

2. Even when the expression of the same gene was modulated by two strains, the level of modulation differs among distinct *H. pylori* strains (eg., VASP, which depending on the strain can be up regulated by as low as 2-fold to as high as 5.2-fold)

3. In addition, these experiments have identified several novel host candidate genes that are modulated during early infection with *H. pylori*. Monitoring novel signal transduction pathways should lead to better understanding of *H. pylori*’s pathogenesis.

Next, I verified PCR-array results, for cytoskeletal regulator VASP which was significantly up regulated (5.26X) in AGS cells following a 6 hr infection with *H. pylori* strain G27MA, using fluorescent activated cell sorting (FACS) analysis that compared VASP expression of uninfected AGS cells and AGS cells infected with *H. pylori* strain G27MA for 6 hr. Confirming the PCR array result, significant increase in VASP fluorescence i.e., VASP expression was observed in AGS cells infected with G27MA as
compared to uninfected AGS cells (Fig. 19D). This assay lends further credibility to the PCR array data and analysis that I performed above.
Figure 19: Differential dysregulation of cytoskeletal regulators following *H. pylori* infection with diverse *H. pylori* strains.
(A) Differential AGS cell scattering by diverse *H. pylori* strains.

Six hours post-infection, images were taken of the various wells containing AGS cells infected with indicated *H. pylori* strains. Cell extensions (arrow heads) measuring over 40 μmeters were considered "scattered" or "hummingbird" phenotype. 100 total cells in each field and three random fields were counted for each experiment. Experiment was done in triplicate and error bars represent standard deviations.

(B) Significant increase in the percentage cell scattering of AGS cells were observed following infection with diverse *H. pylori* strains with Japanese *H. pylori* strain causing the maximum affect.

(C) Molecular basis of cytoskeletal deregulation by *H. pylori*: Cytoskeletal targets are variably affected by diverse *H. pylori* strains.

Scatter plot analysis comparing expression of cytoskeletal regulators following 6hrs of infection with indicated *H. pylori* strains (on y-axis) with their expression in uninfected AGS cells (on x-axis). Number of genes significantly up (≥ 2 fold) and down regulated (< 2 fold) are shown in red and green circles, respectively. Designation of each gene was given right next to its position on the scatter plot.

(D) Significant increase of VASP expression in AGS cells following *H. pylori* G27MA infection.

(i) Depicts a dot plot representation of VASP fluorescence (boxed regions) in uninfected AGS cells and in AGS cells infected with G27MA-WT (ii). Shift of fluorescence to the right of the plot indicates increase in protein expression level; (iii) & (iv) depicts dot plots showing dynamics of CagA and phosphorylated MAPK in uninfected and G27MA infected AGS cells, respectively, which were monitored as controls to ensure the specificity of infection progression. MAPK activation is a hallmark of *H. pylori* infection and is observed only in infected AGS cells.

Summary II

1. HepC is biologically relevant during *H. pylori* infection and genetically diverse *H. pylori* strains tightly modulate hepC expression in AGS cell culture infection model.

2. HepC contributed significantly to *H. pylori*’s fitness.

3. HepC most likely targets host cytoskeletal machinery during late infection in a cell culture model of infection.

4. Genetically diverse *H. pylori* strains differentially dysregulate host cytoskeletal regulators during an early infection of AGS cell culture infection.
DISCUSSION AND FUTURE DIRECTIONS

The ability of *H. pylori* to chronically persist in human gastric mucosa along with striking geographic variation in the clinical outcome of infection suggests *H. pylori* strain specific exploitation and modulation of host responses. Emerging data suggests a possible role for proteins encoded by the *H. pylori* slr genes, in mediating and / or managing *H. pylori* - host interaction [42]. *H. pylori* slr genes encode secreted proteins with homology to the Sel-1 group of eukaryotic regulatory proteins that, through their interaction with other eukaryotic proteins, affect cell proliferation, apoptosis, immune response, and intracellular trafficking [43, 45]. Positive selection plays a dominant role in *H. pylori* slr gene family evolution, such that in any given slr protein different amino acids are favored in different geographical areas, and that the selection intensity is stronger on some slr genes than others in natural *H. pylori* populations [42]. Here, I extended this paradigm to *H. pylori* slr genes, hcpC, and hcpG. hcpC is present in all the natural *H. pylori* populations tested whereas hcpG is either absent, pseudogenized or, exhibited extreme polymorphisms. Different hcpC and hcpG codons evolved at different rates in different populations, although the intensity of selection to diversify is higher in hcpG evolution. Localization of adaptive residues to the molecular HcpC surface suggests that these may affect the affinity or specificity of its interaction with cognate host protein/ (s), fine tuning the host responses. Alternatively, some of the adaptive residues might also be
involved in immune escape, given that HcpC is a secreted and immunogenic molecule [54].

One unique feature of *H. pylori* is its outer membrane association of intrinsic cytoplasmic protein, HspB [132-135]. This feature was attributed to autolysis of bacteria and subsequent adsorption of extracellularly released HspB onto intact live bacteria [137]. However, it cannot be totally discounted that other accessory protein transporters, here HcpC, are involved in this process, given that a consistent defect in HspB surface localization is observed in G27MAΔhcpC mutant strain, in a cell culture infection model. Furthermore, HcpC is able to rescue the HspB surface localization defect exhibited by G27MAΔhcpG mutant strain early (3 hr) in cell culture infection model. Surface association of HspB was shown to induce humoral immune response and inflammation, leading to gastritis [138-143], and can also probably mask the integral intrinsic outer membrane proteins of *H. pylori* from host immune recognition [52, 137, 140, 144]. Thus, my observation has important implications in *H. pylori* pathogenesis by promoting long term survival of *H. pylori* in the host by evading immune surveillance. Exactly how HcpC is involved in surface translocation of HspB now merits detailed analysis. Furthermore, the G27MAΔhcpC mutant, but not the G27MAΔhcpG mutant, exhibited significant relative fitness reduction in a cell culture infection model. However, the combined effect of paralogs, hcpC and hcpG to *H. pylori* fitness is higher than the fitness contributions provided by each paralog individually. Taken together, these findings indicate that HcpC and HepG are genetically redundant, but functionally non-reciprocal, and that HcpC and HepG perform a crucial role in gastric epithelial cell infection. Given
these findings, I propose the following model for emergence, fixation and preservation of hcpG alleles in *H. pylori* genomes via gene duplication from ancestor hcpC (Figure 20).

**Model for evolution of hcpG in *H. pylori* genomes:**

![Model Diagram](image)

**Figure 20:** Evolution of stable, non-reciprocal genetic redundancy by diversifying selection following duplication and divergence in *H. pylori* Sel 1-like gene family.

Explanation of terms used:

- $\Delta$hcpG: Deletion of hcpG;
- phcpG: Pseudogenized hcpG alleles; vhcpG: variant functional hcpG alleles;
- EhcpC: Efficiency of hcpC function; EhcpG: Efficiency of hcpG function;
- $\omega = d_{w}/d_{s}$; $\pi_{n}$ = nucleotide diversity per non-synonymous site; $\pi_{s}$ = nucleotide diversity per synonymous site.

Following gene duplication, the duplicated copy usually undergoes an initial fixation phase where the duplicate achieves fixation in the population if not lost by genetic drift [145]. Fixation phase is followed by the fate – determination phase, where the duplicated copy accumulates fate - determining mutations some of which can then be fixed by natural selection. The final phase is the preservation phase, where the fixed
change/s are maintained in the population [145]. In addition, selection pressures operating on the gene duplicates in each of the phases can significantly contribute to their preservation and functional evolution in genomes [145]. Based on the evolutionary and functional findings on paralogs, hcpC, and hcpG in this study, I propose the following model (Figure 20): Following the duplication event whereby hcpC duplicates to give rise to hcpG, fixation phase begins. In the fixation phase, hcpG is usually lost from the population as a consequence of genetic drift (ΔhcpG). However, if hcpG escapes genetic drift mediated loss, it acquires random mutations in the fate determining phase. Such mutations, if they confer a functional advantage to H. pylori, are fixed in the genomes (vhcpG). If the mutations are not conferring an advantage, they will be pseudogenized (phcpG) and be lost from the populations (ΔhcpG). However, some null mutations that can contribute to the improved fitness of H. pylori, are fixed in the genomes (evidence: retention of phcpG in some H. pylori genomes). Proceeding into preservation phase, positive selection then operates on the duplicated paralog (vhcpG) that accumulated loss of function / degenerative mutations in fate - determining phase (evidence for accumulation of loss of function mutations by hcpG: HcpG is only required for optimal HspB expression during early infection, and is unable to compensate for the lack of HcpC during later phases, whereas HcpC is necessary and sufficient for optimal surface expression of HspB. Furthermore, the contribution of hcpC to H. pylori fitness, in the AGS cell culture infection model, is significantly greater than hcpG. However, both genes together demonstrated an additive effect on H. pylori fitness during 24 hrs post infection; SΔhcpC = 0.264 vs. SΔhcpG = 0.074, P<0.01; SΔhcpC or SΔhcpG vs. SΔhcpC::ΔhcpG = 0.431, P<0.01, where S=coefficient of median fitness reduction). Thus, HcpG
subfunctionalized in HcpC function, and is preserved in *H. pylori* genomes with a stable, genetically redundant, epistatic and overlapping yet non-reciprocal functional relationship with *hcpC*. There is also evidence of positive selection in the evolution of *hcpC* in the preservation phase (*hcpC*; $\omega > 1$). Yet, the selection intensity on *vhcpG* in the preservation phase is much higher than *hcpC* ($\omega_{vhcpG} > \omega_{hcpC}$), indicating that *vhcpG* diversified or is diversifying significantly from the ancestor gene *hcpC* most probably for functional divergence, and that natural selection favored retention of the ancestral *hcpC* likely with an enhanced functional efficiency. Taken together, my data suggests a novel mechanism by which natural selection selects stable redundancy in duplicated genes. So, how does my data not fit in completely in any of the well-established models of gene duplication evolution? Since my data show that both *hcpC* and *hcpG* have evolved by strong diversifying selection in the preservation phase, I will briefly describe the models that take into account that positive selection is involved in the process of stable maintenance or preservation of both the duplicated genes, and explain how my data deviate from those models:

1. Duplication – degeneration – complementation (DDC model) [146]: Here, both the paralogs accumulate degenerative mutations that reduce the functional efficacy of both the duplicated genes. As a result, neither copy is sufficient to perform the original function and hence both the copies subfunctionalize, so that they both must be maintained by selection [146-148]. Moreover, symmetrical levels of polymorphisms and divergence among duplicated paralogs are expected in this model given that, both the paralogs undergo degenerative mutations to reduce the efficiency of the same function [145, 149, 150]. In contrast, here I
show that the fitness contribution of both the paralogs (hcpC and hcpG) together is significantly greater than the fitness contributions of each of the paralogs individually, that even though there is evidence of subfunctionalization in HcpG, natural selection favored retention of the ancestral HcpC function but not subfunctionalization, and that there is asymmetrical levels of non-synonymous levels of polymorphism ($\pi_N$) in the evolution of hcpC and hcpG ($\pi_N^{hcpG} > \pi_N^{hcpC}$).

2. Escape from adaptive conflict model (EAC): This model assumes that, single copy gene ancestral gene carries out distinct functions, and that it cannot improve one aspect of its performance without negatively affecting other aspects (adaptive conflict) [151-154]. This adaptive conflict is resolved when a duplication event gives one of the paralogs a chance to escape one of its roles. Therefore, duplication is accompanied by adaptive mutations in both duplicated genes to fine-tune their newly attained subfunctions, with signatures of positive selection on sequences released from adaptive conflict [151-154]. Moreover, the fitness contributions of both paralogues will be greater than the unduplicated paralog [143] and the polymorphism patterns will be similar to that of DDC model [143].

Even though my data is in harmony with this model with respect to the fitness dynamics and polymorphism patterns of the paralogs, it is the functional aspect where this model differs significantly. I show that instead of each of the paralog (hcpC and hcpG) diversifying and specializing in each of the different functions of ancestral gene, they both contribute to the same function i.e., HspB surface localization, albeit with different efficiencies.
3. Permanent heterozygote model: This model assumes that genetic variation already exists for a gene before the duplication event, that following gene duplication formation and fixation of a permanent heterozygote results in the achievement of higher fitness than either of the homozygotes in the pre-duplication phase, and that high levels of polymorphisms exist before the gene duplication event [145, 155, 156]. To consider this model for hcpG evolution in H. pylori genomes, the main assumption would be that following duplication the permanent heterozygote (here, genomes with both hcpC and hcpG) will be fixed in the populations. My data clearly contradict this assumption in that nearly 50% of H. pylori populations tested lack hcpG.

4. Multi-allelic diversifying selection: The main assumption of this model is that the functional attributes of genes under multi-allelic diversifying selection, requires the genes to evolve constantly and rapidly (e.g., major histocompatibility genes) for functional divergence, and hence positive selection favors fixation of new copy [145, 157, 158]. However, my data show that HcpG is subfunctionalized to HcpC function rather than acquiring a new function.

In an attempt to delineate the function(s) of HcpG, I cloned and expressed the five unique hcpG allelic variants as 6 X-Histidine fusion proteins. Attempts at purifying HcpG::His, to identify the interacting host partner(s) are still in process.

Studies in our lab have determined that H. pylori SLR protein HepC interacts directly with multifunctional human cytoskeletal protein, Ezrin and that the Japanese HepC variant exhibited a greater affinity for Ezrin interaction when compared to its European counterpart, suggesting that such interaction differences likely manifest in
altered Ezrin-dependent signal transduction which may directly influence the progression and thus the severity of gastric disease outcome in Japanese populations (Figure 21). It is in this context that I characterized the biological significance of HepC in \textit{H. pylori} pathogenesis. I showed that the different geographic variants of \textit{hepC} differ in their expression patterns in the AGS cell culture infection model, with Japanese \textit{hepC} exhibiting maximal up regulation following infection. This finding is interesting given that HepC – Ezrin interaction affinity is also the strongest in Japanese \textit{H. pylori} populations. Whether such increased binding affinity can be directly correlated with increased \textit{hepC} expression in Japanese \textit{H. pylori} populations needs to be fully characterized. HepC contributes significantly to \textit{H. pylori}'s fitness and most likely targets host cytoskeletal machinery by inhibiting key components of the human cytoskeletal machinery, during late infection in the cell culture model. It will be noteworthy to elucidate whether such cytoskeletal deregulation is mediated by HepC – Ezrin interaction or, by other yet unidentified mechanisms of HepC – host mediated interactions.

Furthermore, genetically diverse \textit{H. pylori} strains differentially impact key cytoskeletal regulators during relatively early cell culture infection model. Yet more, amplitude of expression of the identical cytoskeletal regulators varies following infection with diverse \textit{H. pylori} strains. It is tempting to consider that such dysregulation of cytoskeletal regulators following infection with diverse \textit{H. pylori} strains can affect the downstream events in two different ways: 1) Dramatic: modulation and alteration of different signaling pathways, 2) Subtle: fine tuning identical signaling pathways. These findings should help develop a population-based framework in understanding molecular events behind geographically variable clinical outcome of gastric disease, and in developing
population specific biomarkers to predict the outcome of *H. pylori* infection. In addition, these experiments have identified several novel host candidate genes that are modulated during early infection with *H. pylori* and thus the need to monitor novel signal transduction pathways to better understand *H. pylori*’s pathogenesis.

**Figure 21:** Causes and consequences of *H. pylori* molecular evolution.

Taken together, of the myriad of positive selection outcomes, I specifically showed that positive selection mediated genetic redundancy provides a fitness advantage to *H. pylori* (Figure 20), and that positive selection mediated modulation of *H. pylori* - host interactions likely alters the strength, duration or amplitude of downstream signaling events (Figure 21). I speculate that some of these differences may affect the progression
of *H. pylori* infection, and thereby the clinical outcome in different geographical regions.

I conclude that, further search for, and analysis of, *H. pylori*’s determinants that have been subject to positive selection in particular lineages should elucidate mechanisms important in establishment and maintenance of chronic infection and disease, and perhaps provide new insights into effective management or eradication of these infections in diverse human populations.
REFERENCES


APPENDIX

I. Confidently predicted domains, repeats and motifs of HepC and HepG in the available *H. pylori* genomes using SMART analysis and Signal IP HMM prediction.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Name</th>
<th>Begin</th>
<th>End</th>
<th>E-value</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Signal peptide</td>
<td>1</td>
<td>25</td>
<td>0.991</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SEL1</td>
<td>104</td>
<td>135</td>
<td>0.0289</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>SEL1</td>
<td>136</td>
<td>171</td>
<td>5.75E+01</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>SEL1</td>
<td>172</td>
<td>207</td>
<td>1.94E-12</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>SEL1</td>
<td>208</td>
<td>243</td>
<td>4.12E-07</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>SEL1</td>
<td>244</td>
<td>279</td>
<td>5.83E-07</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>SEL1</td>
<td>280</td>
<td>315</td>
<td>6.15E-06</td>
<td>NA</td>
</tr>
<tr>
<td>G27_HcpG</td>
<td>Signal Peptide</td>
<td>1</td>
<td>25</td>
<td>0.991</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SEL1</td>
<td>105</td>
<td>136</td>
<td>0.0356</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>SEL1</td>
<td>137</td>
<td>172</td>
<td>4.78E+01</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>SEL1</td>
<td>173</td>
<td>208</td>
<td>1.46E-07</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>SEL1</td>
<td>209</td>
<td>244</td>
<td>6.15E-06</td>
<td>NA</td>
</tr>
<tr>
<td>J99_HcpG</td>
<td>Signal Peptide</td>
<td>1</td>
<td>27</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SEL1</td>
<td>104</td>
<td>135</td>
<td>0.0289</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>SEL1</td>
<td>136</td>
<td>171</td>
<td>5.75E+01</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>SEL1</td>
<td>172</td>
<td>207</td>
<td>1.94E-12</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>SEL1</td>
<td>208</td>
<td>243</td>
<td>4.12E-07</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>SEL1</td>
<td>244</td>
<td>279</td>
<td>5.83E-07</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>SEL1</td>
<td>280</td>
<td>315</td>
<td>6.15E-06</td>
<td>NA</td>
</tr>
<tr>
<td>B8_HcpG</td>
<td>Signal Peptide</td>
<td>1</td>
<td>24</td>
<td>0.991</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SEL1</td>
<td>28</td>
<td>59</td>
<td>2.60E+01</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>SEL1</td>
<td>101</td>
<td>132</td>
<td>0.285</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>SEL1</td>
<td>133</td>
<td>166</td>
<td>0.327</td>
<td>NA</td>
</tr>
<tr>
<td>HpV225_HepG</td>
<td>Signal Peptide</td>
<td>1</td>
<td>25</td>
<td>0.991</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SEL1</td>
<td>29</td>
<td>60</td>
<td>2.34E+01</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>SEL1</td>
<td>61</td>
<td>96</td>
<td>1.98E-09</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>SEL1</td>
<td>97</td>
<td>132</td>
<td>6.1E-10</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>SEL1</td>
<td>133</td>
<td>168</td>
<td>5.31E-10</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>SEL1</td>
<td>169</td>
<td>204</td>
<td>1.18E-07</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>SEL1</td>
<td>205</td>
<td>240</td>
<td>1.36E-07</td>
<td>NA</td>
</tr>
<tr>
<td>G27_HepC</td>
<td>SEL1</td>
<td>241</td>
<td>276</td>
<td>1.82E-08</td>
<td>NA</td>
</tr>
</tbody>
</table>
II. CFU counts obtained in the AGS cell infection model competition assay between G27MA WT and *str* mutants used in the study

<table>
<thead>
<tr>
<th>CFU counts from competition experiments between G27MA WT and G27AhcpC</th>
<th>G27WT</th>
<th>G27DhcpC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0hr</td>
<td>6hr</td>
</tr>
<tr>
<td>Exp’t 1</td>
<td>4080000</td>
<td>6360000</td>
</tr>
<tr>
<td>Exp’t 2</td>
<td>2060000</td>
<td>7440000</td>
</tr>
<tr>
<td>Exp’t 3</td>
<td>970000</td>
<td>1044000</td>
</tr>
<tr>
<td>Exp’t 4</td>
<td>970000</td>
<td>1488000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CFU counts from competition experiments between G27MA WT and G27AhcpG</th>
<th>G27WT</th>
<th>G27DhcpG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0hr</td>
<td>6hr</td>
</tr>
<tr>
<td>Exp’t 1</td>
<td>464000</td>
<td>468000</td>
</tr>
<tr>
<td>Exp’t 2</td>
<td>464000</td>
<td>756000</td>
</tr>
<tr>
<td>Exp’t 3</td>
<td>464000</td>
<td>522000</td>
</tr>
<tr>
<td>Exp’t 4</td>
<td>514000</td>
<td>876000</td>
</tr>
<tr>
<td>Exp’t 5</td>
<td>514000</td>
<td>766000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CFU counts from competition experiments between G27MA WT and G27L1hcpC:L1hcpG</th>
<th>G27WT</th>
<th>G27DhcpC:AhcpG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0hr</td>
<td>6hr</td>
</tr>
<tr>
<td>Exp’t 1</td>
<td>586000</td>
<td>862000</td>
</tr>
<tr>
<td>Exp’t 2</td>
<td>586000</td>
<td>792000</td>
</tr>
<tr>
<td>Exp’t 3</td>
<td>586000</td>
<td>910000</td>
</tr>
<tr>
<td>Exp’t 4</td>
<td>586000</td>
<td>946000</td>
</tr>
<tr>
<td>Exp’t 5</td>
<td>536000</td>
<td>648000</td>
</tr>
</tbody>
</table>
III. CFU counts in *in vitro* competition assays.

<table>
<thead>
<tr>
<th></th>
<th>G27WT</th>
<th>G27ΔhcpC</th>
<th>G27ΔhcpG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0hr</td>
<td>12hr</td>
<td>56hr</td>
</tr>
<tr>
<td>Exp't 1</td>
<td>5640000</td>
<td>16920000</td>
<td>67800000</td>
</tr>
<tr>
<td>Exp't 2</td>
<td>5640000</td>
<td>16200000</td>
<td>70200000</td>
</tr>
<tr>
<td>Exp't 3</td>
<td>5640000</td>
<td>16520000</td>
<td>69800000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>G27WT</th>
<th>G27ΔhcpC</th>
<th>G27ΔhcpG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0hr</td>
<td>12hr</td>
<td>56hr</td>
</tr>
<tr>
<td>Exp't 1</td>
<td>5640000</td>
<td>19200000</td>
<td>83200000</td>
</tr>
<tr>
<td>Exp't 2</td>
<td>5640000</td>
<td>18700000</td>
<td>78600000</td>
</tr>
<tr>
<td>Exp't 3</td>
<td>5640000</td>
<td>19000000</td>
<td>80800000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>G27WT</th>
<th>G27ΔhcpC-G</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0hr</td>
<td>12hr</td>
</tr>
<tr>
<td>Exp't 1</td>
<td>5640000</td>
<td>17000000</td>
</tr>
<tr>
<td>Exp't 2</td>
<td>5640000</td>
<td>17600000</td>
</tr>
<tr>
<td>Exp't 3</td>
<td>5640000</td>
<td>18500000</td>
</tr>
</tbody>
</table>
IV. CFU counts obtained for growth assays of G27MA WT and hcp mutants in AGS cell culture infection model.

<table>
<thead>
<tr>
<th>Growth dynamics of G27WT and G27 - hcp mutants in an AGS cell infection</th>
<th>G27WT</th>
<th>G27 Δ hcpG</th>
<th>G27 Δ hcpC</th>
<th>G27 Δ hcpC Δ hcpG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp'1</td>
<td>0hr</td>
<td>6hr</td>
<td>24hr</td>
<td>0hr</td>
</tr>
<tr>
<td>Exp'2</td>
<td>2300000</td>
<td>4460000</td>
<td>1150000</td>
<td>2280000</td>
</tr>
<tr>
<td>Exp'3</td>
<td>2820000</td>
<td>4920000</td>
<td>1260000</td>
<td>2280000</td>
</tr>
<tr>
<td>Exp'4</td>
<td>2820000</td>
<td>4860000</td>
<td>1620000</td>
<td>2780000</td>
</tr>
<tr>
<td>Exp'5</td>
<td>2980000</td>
<td>4120000</td>
<td>1840000</td>
<td>3040000</td>
</tr>
</tbody>
</table>

V. Assessment of RNA quality for PCR arrays.

(A) The assessment of RNA integrity with Agilent 2100 bioanalyzer shows the electropherogram of extracted RNA samples (1 – 10) used in PCR arrays. (B) RIN visualization using the Agilent expert software for representative samples from lanes 2 & 5.
Curriculum Vitae

Kalyani Putty

139 Life Sciences Building
Department of Biology
Address and University of Louisville
Contact Information Louisville, KY 40292
Phone: 502-287-2426
EMail: kalyaniputty@gmail.com, k0putt01@louisville.edu

A. Education

Dec 1999-Jan 2005

Bachelors of Veterinary Science and Animal Husbandry
(B.V.Sc.&A.H); equivalent to Doctor of Veterinary Medicine
(D.V.M) Acharya N.G.Ranga Agricultural University,
Hyderabad.

Jan 2006-Present

Working towards Ph.D (to be graduated in Dec 2010) in Dept of
Biology, Division of Molecular Cellular and Developmental
Biology, University of Louisville, Louisville, KY.

B. Positions Held


Junior Veterinary Resident, Veterinary Poly clinic, Guntur.

Jan. 2006 – Present

Graduate Teaching Assistant, Dept of Biology, University of
Louisville, Louisville, KY.

C. Academic and Professional Honors

May 2009

Awarded Centre for Genetics and Molecular Medicine (CGeMM)
Travel award for attending/presenting at 109th American Society
of Microbiology (ASM) Conference, Philadelphia, PA.

D. Research (Jan 2006 – Dec 2010)

My dissertation research focuses on understanding the pathogenesis of gastric pathogen
*Helicobacter pylori* and the role of its genetic diversity in geographical differences seen
in the clinical outcome of the *H. pylori* infection.
i. Works Published


ii. Work completed, Manuscripts to be submitted.


NOTE: completed work presented in part at “Posters at the Capitol”, Frankfort, KY.

iii. Presentations at International and National Scholarly Meetings.

1. Putty K, Marcus SM, Berg DE and Kalia A. 2006. Geographic subdivision facilitates adaptations in Helicobacter pylori populations; Pathogen evolution in adaptive landscapes In, Research Louisville, Louisville, KY.


iv. Research Interests.

My research interests in a broad sense are: 1) To understand the mechanisms of bacterial pathogenesis, 2) Identifying and developing molecular targets for therapeutic purpose and vaccine preparations.

E. Teaching (Spring 2006 – Fall 2010)

i. BIOL104 (Laboratory for Introduction to Biological Systems – 1 credit hour)
This course provides hands-on laboratory experience for non-science students and is designed to develop skills in scientific methodology, observation, and critical thinking.
Role: Teaching Assistant.

ii. BIOL258 (Introduction to Microbiology Laboratory - 1 credit hour)
This course teaches basic microbiological techniques that are necessary to study and safely handle microorganisms and viruses.
Role: Lead Teaching Assistant.

iii. BIOL400 (Histology Lab – 4 credit hours)
This course targets pre-medical students and teaches microscopic anatomy and function of vertebrate tissues and organs.
Role: Teaching Assistant.