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L-LACTATE BRIDGES *HELICOBACTER PYLORI* AND ITS HOST

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by

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ABSTRACT

Lactate bridges *Helicobacter pylori* and its host

By

Shuai Hu

The complement system has long been appreciated for its role in bloodborne infection, but its activities in other places, including the gastrointestinal tract, are unknown. Here, we report that complement restricts gastric infection by the pathogen *Helicobacter pylori*. This bacterium colonized complement-deficient mice to higher levels than wild-type counterparts, particularly in the gastric corpus region. *H. pylori* used uptake of the host molecule L-lactate to create a complement-resistant state that relied on blocking the deposition of the active complement C4b component on *H. pylori*'s surface. *H. pylori* mutants unable to achieve this complement-resistant state had a significant mouse colonization defect that was largely corrected by mutational removal of complement. This work highlights a previously unknown role for complement in the stomach, and has revealed a new mechanism for microbial-derived complement resistance.

We further found that to mediate complement resistance, L-lactate played a role as signaling molecule that mediated the upregulation of a small regulon, including the phospholipase A (PldA) coding gene. PldA was critical to prevent killing by complement activation. PldA significantly enhanced *H. pylori* complement tolerance by efficiently dissociating C4b on the *H. pylori* surface. Without PldA, *H. pylori*

survival was impaired under complement exposure, and mutants were unable to colonize mouse stomachs. This work highlights a previously unknown function of phospholipases in complement resistance, and a new type of bacterial anti-complement mechanism. Our results suggest PldA may plays essential role in facilitating *H. pylori* gastric colonization through facilitating complement resistance.

Last, to conduct the investigations, we applied genetic manipulation to generate isogenic mutants from WT *H. pylori* background. Genetic manipulation is a frequently applied approach to study numerous bacterial processes, including *H. pylori*. However, *H. pylori* is difficult to manipulate, partially due to robust Restriction-Modification (RM) systems that destroy exogenous incoming DNA. To overcome RM barrier, we developed an easily appliable approach to improve *H. pylori* transformation efficiency. For this approach, we computationally predicted under-represented short Kmer sequences in the *H. pylori* genome, with the idea that these sequences reflect restriction enzyme targets. We then used this information to modify an antibiotic resistance cassette by generating synonymous mutations at the predicted restriction sites, and use this modified cassette for transformation. Indeed, antibiotic cassettes with modified under-represented Kmer sites resulted in up to 10^5-fold higher transformation efficiency compared to a non-modified cassette. Because our approach relies on computational Kmer site prediction, it is readily applicable to any microbe with a sequenced genome. We thus expect this approach will enable genetic manipulation to be more achievable in a wide range of bacterial species.

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CHAPTER 1

Lactate: emerging battle center between host and pathogens

1.1 Abstract

Lactate is one of best known end product of glycolic fermentation. Lactate production increases in response to intensive energy demands. Lactate functions as a metabolic waste product, but has also been suggested to serve additional roles in different organisms. In mammals, lactate homeostasis is important to body health and lactate production regulates multiple cellular activities. In microorganisms, lactate supports microbial proliferation and promotes their virulence at different aspects. It can thus be view that microbes promote lactate for growth and virulence, while the host limits it to keep pathogens under control. In this review, we summarize emerging evidence and discuss this interaction in which lactate regulates host activities and promotes microbial adaptation in the host environment. Based on current knowledge, we provide a perspective that lactate concentration impacts the outcome of host and microbial interplay as the host tends to tightly regulate lactate fluctuation between 1-2 mM while pathogens attempt to maintain high level of lactate.

1.2 Introduction

Nutritional immunity is a popular topic in the study microbial and host interaction. In terms of the functionality, many nutrients that are used in similar ways by both prokaryotic and eukaryotic cells. It has been realized that serious battles happen for over the acquisition of those nutrients (Olive & Sassetti, 2016; Sheldon et al., 2019). Typically, these critical nutrients are amino acids, lipids, Fe, Zn, and so on. Some atypical nutrients were recently found beyond those classical ones. For example, the metabolite lactate was previously recognized as a waste product from the glycolysis-

fermentation pathway. Recently, lactate was realized to play multiple roles in both host and microorganisms. In the host, lactate is a carbon fuel that can be used by various cell types (Rabinowitz & Enerback, 2020), participating in energy and immune regulation, mediating wound healing, and even involving in cancer development and metastasis. In the microorganisms, lactate promotes cellular proliferation and signals microbes to upregulate immune defense. Compared to the classical nutrients that are tightly controled by host, lactate is commonly released extracellularly and widely distributed. All these functions allow lactate to intimately interact with both host and invading pathogens. In this review, we discuss emerging ideas for how lactate regulates both host and microbial activities and how both of these entities control lactate production.

1.3 Lactate production reflects host cellular activities

Mammals acquire energy through oxidative phosphorylation (OXPHOS) as the major way. During this process, one glucose is broken down into two pyruvates, and further converted into acetyl-CoA to enter tricarboxylic acid (TCA) cycle for full oxidation. This process allow mammals to extract maximum usable energy from one molecule of glucose. Alternative processes occur when ATP demand exceeds the supply from oxidative respiration. In such condition, pyruvate, the end product of glycolysis, can be directly reduced to lactate through lactate dehydrogenase (LDH) instead of entering into TCA cycle, a process called fermentation (Rabinowitz & Enerback, 2020). Fermentation is the preferred metabolic path in some cell types under high proliferation status, including cancer cells and stem cells (Sancho et al., 2016).

Some other cell types, like macrophages, are able to reprogram their metabolism from OXPHOS to fermentation in response to various damage-associated molecular patterns (DAMPs) (Kelly & O'Neill, 2015). Lactate fermentation is 50-100 times faster than OXPHOS and so can rapidly produce ATP. For this reason, mammal cells operate this pathway to satisfy intensive energy demands. Although fermentation is well known to be oxygen-independent, such a metabolic shift in the presence of oxygen, called the Warburg effect, is a hallmark of cancer development (Liberti et al., 2016).

Mammals employs various signaling pathways to activate the Warburg effect. Some transcription factors, like hypoxia-inducible factor-1 alpha (HIF-1α), β-catenin, and kappa-light-chain-enhancer of activated B cells (NF-κB), are commonly involved to sustain this effect (Taylor & Colgan, 2017; Zhang & Wang, 2020; D'Ignazio et al., 2017). These transcriptional factors can be activated in variety of ways. For example, HIF-1α is a transcriptional factor that is up-regulated when cells are under hypoxic condition, commonly reported in cancer cells. HIF-1α has an intrinsically short half life under normoxic conditions since it is rapidly degraded by the ubiquitin-proteasome system (Salceda & Caro, 1997). However, HIF-1α stabilization has been reported to occur during inflammation in different immune cell types, such as myeloid cells, dendritic cells, and natural killer T-cells (McGettrick & O'Neill, 2020). Stabilized HIF-1α is active to regulate metabolic reprogramming by inducing several key players that promote the fermentation pathway (Taylor & Colgan, 2017). The up-regulated genes include glucose transporters (GLUTs) and hexokinase (HK) 1 and 2 that are responsible for increasing glucose transportation (Yang et al., 2019), lactate dehydrogenase A

(LDHA) that converts pyruvate to lactate (Feng et al., 2018), and the monocarboxylate transporter 4 (MCT4) that transports lactate to the extracellular space (Ullah et al., 2006). In addition, HIF-1α is able to up-regulate pyruvate dehydrogenase kinase (PDK1) that blocks the formation acetyl-CoA by enzymatically inhibiting pyruvate dehydrogenase (Kim et al., 2006), which in turn shunts available pyruvate towards lactate formation. In addition, HIF-1α was found to be regulated by NF-κB. NF-κB is a master transcriptional regulator that promotes and is upregulated during inflammatory responses, as reviewed elsewhere (Ghosh & Hayden, 2008, Hayden & Ghosh, 2012). Interestingly, its subunit p50 and p65 can bind to the HIF-1α promoter region that up-regulate HIF-1α. Thus, inflammatory conditions with high NF-κB promotes HIF-1α expression even in normoxic condition (van Uden et al., 2008).

Abundant evidence has suggested that various signaling pathways can be independently or crosslinked to reprogram cellular metabolism, and lactate production is the outcome of all these complex cellular activities. However, the activation of these pathways exhibit distinct spatial and temporal patterns. For example, β-catenin is another active transcriptional factor that is commonly reported in stem and cancer cells (Zhang & Wang, 2020). β-catenin and HIF-1α promotes cancer development through up-regulating glycolysis and lactate fermentation. β-catenin in other cells, such as Batf3-lineage $CD103^+$ dendritic cells, can induce transcriptional repressor ATF3 which blocks the production of CCL4 to further reduce the infiltration of $CD8^+$ T cells. β-catenin also promote Treg responses in $CD4^+$ T cells, supporting an important role in limiting the immune response. More importantly, β-catenin is commonly involved in

angiogenesis and would healing process after tissue injury (Li et al., 2019), suggesting that β-catenin may play important role in late stage of inflammatory responses. One idea is that the β-catenin triggers a cell physiology that utilizes lactate fermentation to support tissue regeneration. Combining all these evidence, lactate overproduction occurs via several cell signaling pathways that in turn reflect the status of tissue-specific activities.

1.4 Lactate regulates inflammatory responses

Inflammatory responses are a significant component of the host response to microbial infection. Inflammation includes a large set of responses that are both pro-inflammatory and anti-inflammatory. These responses vary in a temporal and spatial pattern, and lactate plays important roles to inversely regulate those activities. At the initial stage, the host responds the bacterial infection and activates inflammatory activities. Fermentation is also activated to satisfy the intensive energy demand. For example, murine bone marrow-derived neutrophils rapidly induce glycolysis and accumulate lactate in response to the presence of *Salmonella typhimurium infection or LPS stimulation (Khatib-Massalha et al., 2020).* Local lactate accumulates from <2 mM at homeostatic condition to 10-20 mM (Libre et al., 2021). Lactate's role in inflammatory responses at the initial stage is not yet known, but apparently the low initial level of lactate is not sufficient to block immune activation.

Continuous high lactate has a well-documented regulatory role on inflammatory responses. A recent study found that elevation of lactate can decrease

glucose uptake and be converted back to pyruvate through LDH in activated $CD4^+$ T cells, consequently resulting an inhibitory feedback on glycolysis (Certo, 2020). Studies have also demonstrated that activated immune cells can be inhibited by lactate treatment (Manoharan et al., 2021). Interestingly, all these studies used high lactate concentrations, above 10 mM, to be able to observe a clear inhibitory role of lactate, suggesting that lactate immune regulation may depend on its local concentration.

Besides the nutritional feedback, lactate is capable of being a signaling molecule to suppress host inflammatory responses. Recently, Zhang *el al*. discovered non-metabolic functions of intracellular lactate during disease development. Lactate itself can act as a precursor for a epigenetic modification called lactylation. Lactylation of histone lysine residues in M1 macrophages directly activates target genes transcription. With accumulation of intracellular lactate, histone lactylation level increases and induces the up-regulation relevant genes expression for homeostasis recovery (Zhang et al., 2019). This action facilitates the inflammatory termination and the recovery to homeostatic status. Besides acting as intracellular signaling molecule, extracellular lactate is also able to function as a signaling molecule to regulate inflammatory responses from over activation and protect from organ damage. One response occurs when lactate activates the receptor G-protein-coupled receptor 81 (GPR81) signaling pathway. GPR81 activation downregulates the downstream factor cyclic AMP (cAMP) and protein kinase A (PKA), which in turn inhibit lipolysis to block immune activation in fat cells (Hu et al., 2020). This response happens at physiological concentration (0.2-1 mM), suggesting it could occur after acute immune

activation. Taken together, these studies support the idea that lactate acts as a signaling molecule to prevent over activation of inflammatory responses.

Overproduced lactate will be eventually recycled in the liver through the gluconeogenesis pathway (Rabinowitz & Enerback, 2020). Lactate production and inflammatory response looks like a parabolic curve. At low levels, lactate does not inhibit inflammation. With elevated concentrations, lactate exhibits a regulatory role, inverse to its concentration; high lactate inhibits inflammatory responses. The high lactate will then be recycled in the liver to return concentrations to basal level. In sum, lactate production in host immune system is tightly regulated, as the host produces high levels during critical energetic needs, and then recycles it once immune activities are ended.

1.5 Microbial pathogenesis is associated to altered host metabolism

Various pathogens have been found to positively manipulate host signaling pathways, activating glycolysis-lactate fermentation pathway. Currently, lactate has been realized as an important carbon and energy source for various pathogens that colonize diverse niches, including *Neisseria meningitidis*, *Neisseria gonorrhea*, *Staphylococcus aureus*, and *Salmonella enterica* serovar *Typhimurium*, *Chlamydia pneumoniae*, *Brucella abortus*, *Legionella pneumophila* (Gillis *et al.*, 2018; Jiang *et al.*, 2014). These findings suggest lactate utilization may be a widespread strategy for pathogens to survive, adapt, and grow in the host microenvironment. Accordingly, lactate utilization related enzymes have been widely investigated. Transport of lactate

into cytoplasm depends on lactate permease (LctP) and its oxidation to pyruvate relies on lactate dehydrogenase, either NAD-dependent or NAD-independent, as clearly summarized by Jiang and colleagues (Jiang et al., 2014).

1.6 Microbial pathogens manipulate host signaling pathways to promote lactate production

Various pathogens positively manipulate host signaling pathways, with lactate production as one of the significant outcomes. Here, we discuss how lactate could be induced by some well-studied pathogens.

The gastrointestinal (GI) tract is a home for a large amount of microbial organisms. In the gastric region, a recent study found that *Helicobacter pylori*, a dominant chronic pathogens in the stomach and one of the main risk factor for gastric cancer, preferentially utilizes carboxylic acids and amino acids during colonization, including lactate (Keilberg et al., 2021). From our examination, we found that local lactate was significantly induced in gastric tissue during *H. pylori* infection (Supplemental Figure 1, Chapter 2), The source of lactate has not been definitively identified, but the two main virulence factors CagA and VacA may play an essential role in lactate creation. For example, increased epithelial proliferation was observed during *H. pylori* colonization, dependent on CagA (Peek Jr. et al., 1997, 1999). Epithelial proliferation is promoted by the CagA-activated ERK/MAPK pathway. Specifically, CagA initially binds the growth factor receptor bound 2 (Grb2) to further bind the son of sevenless (SoS), which promotes the formation of Ras-GTP complex

to substantially activate the Raf-MEK-ERK pathway (Gale et al., 1993; Masanori, 2004; Mimuro et al., 2002; Selbach et al., 2009). On the other hand, unphosphorylated CagA can bind to E-cadherin to prevent the E-cadherin/β-catenin complex formation (Murata-Kamiya et al., 2007). This action, together with VacA that inhibits GSK3β through PI3K/Akt signaling, releases β-catenin to cytoplasm and translocates it to the nucleus. β-catenin upregulates pyruvate dehydrogenase kinase, which blocks acetyl-CoA formation. In addition, *H. pylori* was observed to induce transcription factor HIF-1α with PI3K and mTOR dependent manner (Canales et al., 2017). HIF-1α is also capable of promoting glycolysis, as discussed above. β-catenin and HIF-1α together are able to push glycolysis towards lactate production. Thus, *H. pylori* utilizes lactate and has sophisticated strategies to activate host signaling pathways that lead to epithelial cell proliferation and concomitant lactate production.

A similar phenomenon was also found in the intestine. *Salmonella typhimurium* (*S*. Tm) triggers host inflammatory responses and induces dysbiosis in the host intestine. The dysbiosis consists of depleted butyrate-producing Clostridia, which leads to an inhibition of the host PPARγ pathway. These actions switch host metabolism from oxidative respiration to lactate fermentation, which causes lactate over-production and creation of excess oxygen. These conditions promote *S*. Tm aerobic respiration, using lactate as electron donor and oxygen as electron acceptor (Gillis et al., 2018). Under these conditions, *S.* Tm outcompetes the microbiota.

Beside colonizing epithelial surfaces, some pathogens colonize within epithelial or immune cells, such as macrophages, to obtain significant protection from various

stresses. Once internalized, many intracellular pathogens were found to positively interact with and alter mitochondrial morphology, localization, and function. These alterations hijack host metabolism and switch to an attenuated tricarboxylic acid metabolism, with reduced oxygen consumption and increased lactate production. Such cellular metabolism modulation has been observed during cell infection by several bacteria, including *L. pneumophila*, *C. trachomatis* (Escoll et al., 2017; Kurihara et al., 2019). Such manipulation is principally important for some bacteria that predominantly use certain carbon sources. *Brucella abortus* is one of such bacteria that invades host phagocytes to cause chronic infection. Relying on host metabolism manipulation, it uses lactate as the sole carbon and energy source for normal survival and proliferation (Czyz et al., 2017). Noticeably, lactate induction could be required for pathogens' survival and persistence, but not simply as a food. For example, Kaposi' sarcoma (KS) is one of the most commonly reported tumor in AIDS patients of Africa. KS-associated herpesvirus (KSHV) is the etiologic agent of KS. It was shown that Warburg-like manipulation is required for KSHV's latent infection in endothelial cells, since LDH inhibitor treatment, which blocks lactate production, causes cell death of KSHV-infected cells but not uninfected neighbor cells (Delgado et al., 2010).

1.7 Lactate induction provides multiple fitness advantages during microbial infection

Invading pathogens acquire additional fitness advantages from lactate induction beyond its use as a carbon source. These include tolerance to inducible reactive Nitric species (RNS), reactive oxygen species (ROS), promotion of biofilm formation, and

resistance to complement-mediated killing. Each of these lactate-responses are discussed below.

1.7.1 Anti-complement

The complement system plays a vital role in restricting microbial infections. It is highly prevalent in blood, and therefore is well known for its protective role in this setting. Bloodborne pathogens have developed numerous anti-complement mechanisms to facilitate their infections. One report demonstrated that bacterial pathogens can utilize environmental cues to activate anti-complement genes. *N. meningitidis* was found to utilize lactate to mediate a defense response to the alternative complement pathway, in a uptake dependent manner (Exley et al., 2005). Through this process, sialic acid biosynthesis was promoted to evade from alternative complement pathway. As a result, *N. meningitidis* extended the bacterial blood infection stage (Exley et al., 2005).

The role of complement system in other body places, such as the gastrointestinal tract, remains unknown. In our own work described in this thesis, we made use of mice lacking complement and determined that complement limits *H. pylori* numbers. Without it, *H. pylori* colonized to unusually high levels (Unpublished data). Given *H. pylori*'s ability to establish chronic infections, the bacterium was speculated to have evolved strategies to diminish complement's detrimental impact. We discovered an anti-complement mechanism that differs from those used by other pathogens. Other microbes block proteolytic steps in complement activation, but we found that *H. pylori* instead prevents stable surface accumulation of a key complement component, C4b, an

activated product from C4 cleavage. Without this accumulation, complement does not lyse *H. pylori*. Lactate upregulated *H. pylori*'s ability to resist complement. With further exploration, lactate was recognized as a signaling molecule to regulate expression of several metabolic and virulence relative genes. One of the up-regulated genes, phospholipase A (*pldA*), was responsible for destabilizing C4b from *H. pylori* surface. Currently, the mechanism about C4b destabilization is not known. PldA is an enzyme that cleaves ester bonds formed via alcohol group and hydroxyl group hydrolysis. We hypothesize that PldA either cleaves potential *H. pylori* molecules that bind to C4b, or cleaves the ester bonds directly formed by C4b and target molecules. Considering that the role of complement in the GI is understudied, this work highlights the importance of complement during the interplay between host and microbes. Understanding complement-pathogen interactions in the GI tract will lay the groundwork for novel ways to eliminate GI pathogens and may contribute to schemes that promote colonization of customized microbiota.

1.7.2 Resistance of reactive nitric species

Reactive nitric species (RNS), such as radical NO, is produced by inducible nitric oxide synthase (iNOS) in wide range of cells and tissues. Induced cytokines, such as interferons, and invading pathogens are both sufficient to activate iNOS. RNS display strong anti-microbial properties by disrupting microbial metabolism, DNA replication, and lipid integrity (Tharmalingam et al., 2017). RNS has these effects by binding to transition metals, organic radicals, thiols, amines or hydroxyl aromatic

groups. Considering the large portion of thiol-containing enzymes and high levels of iron in bacterial respiratory chains, RNS is able to effectively and fundamentally impact bacterial survival. Bacteria have exhibited various strategies to counteract RNS detrimental impact. Not surprisingly, various microbial organisms have enzymes that specifically detoxify RNS, such as Flavohemoglobin (Fhb), S-nitrosoglutathione (GSNO) reductase, and NO_2/NO_3 reductases. The detoxification mechanism of these enzymes have been summarized previously (Poole, 2020; Broniowska, 2013; Vázquez-Torres, 2016).

Beside the equipped anti-NOS mechanism, bacteria can respond the environment to trigger "dormant" gene expression to gain the function to resist NO. One important evidence came from an *Staphylococcus aureus* infection study. In response to phagocyte-produced NO, *S. aureus* up-regulated NO-inducible L-lactate dehydrogenase (*ldh1*) (Richardson et al., 2008). Under normal fermentative conditions, *S. aureus* generates energy and recycles NAD through ethanol production, as its alcohol dehydrogenase (ADH) has higher oxidizing power than LDH. However, this fermentation process is impaired with NO exposure because ·NO inactivates pyruvate dehydrogenase (PDH) that blocks the generation of acetyl-CoA and the further conversion to ethanol. Therefore, to compensate, L-LDH is induced to use NADH as electron donor and pyruvate as electron acceptor with L-lactate as the end product. This study highlights the alternative metabolic adaptation of nitrosative stress to alleviate detrimental NO effect to microbial metabolism (Richardson et al., 2008).

1.7.3 Regulate biofilm formation

Various microbial pathogens perform biofilm style growth, in the form of microcolonies, to facilitate host environment adaptation. *Neisseria meningitidis*, for example, forms microcolonies in the upper respiratory tract, which are crucial for disease development. By disseminating from microcolonies, *N. meningitidis* has been prepared for colonizing new sites and overcoming cell barrier. Lactate was recently found to be involved in regulating biofilm assembly and dissemination. Host derived lactate has been shown to promote the *N. meningitidis* microcolony dispersal process by mediating type IV pili (Tfp) retraction (Sigurlásdóttir et al., 2017, 2021).

1.7.4 Regulating cell surface decoration

Some fungi, such as *Candida albicans*, can efficiently evade immune detection by using lactate to modulate their surface structures. β-glucan, a key pathogen-associated molecular pattern (PAMP) located on the cell surface of *C. albicans*, can be recognized by the receptor Dectin-1 of macrophages and dendritic cells to trigger the phagocytosis and induce inflammatory responses(Ma & Underhill, 2013). To avoid potential recognitions, *C. albicans* has developed defensive mechanisms using lactate to alter surface decoration. Lactate acts as a signaling molecule to activate the evolutionarily conserved receptor Gpr1 pathway and further recruit transcriptional factor Crz1 to control the expression of cell-wall-related genes. With such decoration, β-glucan is masked and becomes invisible to the immune system (Ballou et al., 2016).

1.7.5 Promoting regulatory cytokines

A recent study has found that some bacteria utilize their own lactate as an immunoregulatory molecule to develop successful colonization. *S. aureus* actively produces lactate to increase local lactate level. Elevated lactate was shown to inhibit histone deacetylase 11 (HDAC11). This event in turn increases the histone 3 acetylation and causes unchecked HDAC6 activity at IL-10 promoter region in myeloid-derived suppressor cells (MDSCs) and macrophages. Through this action, IL-10 is produced, and helps to sustains *S. aureus* growth during prosthetic joint infection (PJI) (Heim, 2020).

1.8 Pathogens tend to maintain high level of lactate at the site of infection

Cancer development occurs as an outcome of some bacterial induced chronic infection. Gastric and colon cancers have been found tightly linked to some bacteria chronic infection, such as *H. pylori*, *S. typhimurium*, *E. coli*, and *Fusobacterium* (Duong et al., 2019). Here we use *H. pylori* and *S. typhimurium* as example to discuss the profile of lactate during these two bacterial chronic infections. Considering that *H. pylori* and *S. typhimurium* acquire significant benefit from lactate induction, these bacteria may actively maintain high lactate level during their colonization. Interestingly, such high level of lactate phenomenon has been observed in both gastric and colonic cancer models, which supported this hypothesis. The upregulation of glycolysis in host cells is a critical response to bacterial infection as it promotes the activation of immune cells. This response, however, brings a significant negative influence from high lactic acid production. In some studies, local lactate level can be up to 40 mM (Colegio et al.,

2014), which causes decreases in extracellular pH, called acidosis. Chronic exposure of normal cells to an acidic microenvironment commonly results cellular apoptosis or necrosis in a p53 and caspase-3 dependent manner (Park et al., 1999). The apoptotic phenotype is recognized as an early phase of carcinogenesis. In another aspect, along with lactate production, cellular glucose consumption rate is also largely elevated. To respond such increasingly limited resource, normal cells are stimulated to increase glucose uptake capacity via upregulation of the glucose transporters (GLUTs) and to utilize lactate as fuel in peripheral cells. These two phenotypes are typical cancer feature and observed in both gastric and colonic cancers.

1.9 Conclusion

From current studies, we conclude that host tightly regulates lactate production to meet the requirement of intensive energy demand but avoid high rate of lactate production as a long term activity because of the potential detrimental effect, acidosis and immune suppression. In the other aspect, bacteria tend to induce lactate production for growth and to acquire fitness advantages either by actively stimulating host inflammatory responses, or manipulating host signaling pathways. At the end of inflammatory responses, lactate levels return to homeostatic status, along with the clearance of invading bacteria. While, chronic pathogens are able to constantly manipulate host signaling pathways to force host cells maintain a high glycolysis status. In turn, it provides host an opportunity to develop to some types of carcinogenesis.

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CHAPTER 2

Helicobacter pylori initiates successful gastric colonization by utilizing L-lactate to promote complement resistance

2.1 Summary

The complement system plays key roles in preventing bacterial infections, with components distributed throughout mammals. Studies are limited, however, on complement's role in gastrointestinal defense. Here, we report that complement plays a previously unappreciated role in restricting gastric infection by the pathogen *Helicobacter pylori*. This bacterium colonized complement-deficient mice to higher levels than wild-type counterparts, particularly in the gastric corpus region. *H. pylori* used uptake of the host molecule L-lactate to create a complement-resistant state, that relied on blocking the deposition of the active complement C4b component on the *H. pylori* surface. *H. pylori* mutants unable to achieve this complement resistant state had a significant mouse colonization defect that was largely corrected by mutational removal of complement. This work highlights a previously unappreciated role for complement in the stomach, and has revealed a new mechanism for microbial-derived complement resistance.

2.2 Highlights

- Complement is active in the stomach to restrict bacterial infection
- *H. pylori* adopts a complement resistant state after exposure to L-lactate
- Complement resistance requires L-lactate uptake

- *H. pylori* resists complement by preventing C4b surface deposition
- L-lactate utilization plays a major role in vivo to mediate resistance to complement-directed elimination.

2.3 Introduction

Animals have multilayered mechanisms to combat pathogen exposure. The gastrointestinal (GI) tract in particular experiences regular exposure to numerous microbial species, and therefore employs multiple strategies to restrict colonization by the pathogens in this mix. These restrictions include mechanical barriers, competition from other microbes, and innate immune mechanisms. One critical non-cellular innate immune mechanism is the complement system. It remains unknown whether this system plays vital defensive role in the GI tract and whether GI microbes have distinct ways to deal with this challenge.

The complement system plays crucial roles in defending microbial infection by inducing numerous immune responses, such as direct target cell lysis through formation of membrane attack complex (MAC); recruitment, activation, and regulation of phagocytic cells via anaphylatoxins release; and modulation of adaptive immune responses (Hess, 2016; Merle, 2015a, 2015b; Ricklin, 2010). Complement components are a set of proteins that are mainly produced in the liver, and released into the blood where they travel to tissues throughout the body. Because of its prevalence in the blood, complement is well-known to play critical roles in bloodborne defenses. The function of the complement system in GI tract defense, in comparison, has been relatively understudied, partially because only a few complement components are detectable in

the GI lumen of healthy hosts (Kopp, 2015). Complement components have been detected in the GI tract during chronic diseases, including Crohn's disease, ulcerative colitis, and chronic gastritis (Halstensen, 1990, 1992; Berstad, 1997). These findings suggest complement proteins are present in the GI tract and may play unappreciated roles.

The complement system is set in action by one of three triggering pathways, called classical, lectin, or alternative. Each one is triggered by a different molecule, e.g. classical is triggered by the Fc portion of antibodies protruding from the targeted pathogen, while lectin is triggered by specific surface carbohydrates. After the complement system is activated, it sets off a cascade of proteolytic reactions that process pre-proteins to their active forms (Fig. 1A). In the classical and lectin pathways, a key step is the processing of C4 to its products C4a and C4b, which in turn catalyze the activation of C3 to C3b. C3 conversion to C3b is a central step in the alternative pathway as well. Multiple proteolytic steps follow the C3 step, to eventually result in multiple products and MAC formation.

Many microbes infect the GI tract, suggesting they have developed sophisticated strategies to overcome innate immune restrictions. One pathogen that is well known for its ability to establish and maintain GI colonization is *Helicobacter pylori*. *H. pylori* infects the stomach and is a leading cause of gastritis, peptic ulcer disease, mucosa-associated lymphoid tissue (MALT), and gastric adenocarcinoma (Marshall, 1995; Uemura et al., 2001; Peek, 2002). Sporadic observations have suggested the interplay between complement and *H. pylori*. For example, C3b has been

seen coating some *H. pylori* in human gastric biopsies (Berstad, 1997). Two additional studies suggest *H. pylori* can thwart the action of complement using host proteins, CD59 and vitronectin, that normally protect host cells from complement destruction (Richter, 2016; Rautemaa, 2001). These known *H. pylori* resistance mechanisms rely on blocking late stages of complement activation through recruiting host proteins, but there are not any known *H. pylori* self-produced complement evasion mechanisms unlike found in other microbial species (Freeley, 2016). Thus, there are hints about *H. pylori*'s interaction with complement but the nature of these interactions have not yet been elucidated.

Many pathogens sense aspects of the host environment and upregulate defensive mechanisms (Johnson, 2018; Karkhah, 2019; Rolig, 2011). In the case of complement resistance, it is well known that the host molecule L-lactate promotes complement resistance in *Neisseria meningitidis by activating a* sialic acid biosynthesis pathway that protects against complement's action (Exley et al. 2005). We were intrigued by this response, because L-lactate is known to be important for *H. pylori*. *H. pylori* senses L-lactate as a chemoattractant, directing its migration toward this signal (Machuka et al, 2017). In addition, L-lactate is one of the most depleted chemicals when *H. pylori* grows with gastric organoids, and can be taken up and used for growth (Keilberg, 2021; Takahashi, 2007; Iwatani, 2014). Given lactate's importance, we asked whether it similarly stimulates an anti-complement mechanism in *H. pylori*.

In this study, we explored the importance of the complement system in gastric defense and the mechanisms used by *H. pylori* to avoid it's action. We discovered that

the complement system restricts *H. pylori* proliferation in the murine stomach, in a region specific manner. *H. pylori* employs L-lactate, in turn, to create a bacterial state that inhibits activation of the classical/lectin complement pathway; L-lactate uptake is critical for this response and required in vivo. Finally, we uncovered that this resistance is accomplished in a previously undocumented manner by destabilizing the interaction between the C4b product and the *H. pylori* surface. Overall, our results reveal that complement plays an important role in controlling bacterial colonization in the GI tract, with GI tract bacteria utilizing previously undescribed mechanisms to overcome this challenge.

2.4 Results

2.4.1 Complement is important in gastric defense.

Our first goal was to characterize the role of complement in bacterial stomach colonization. We made use of mice that lack the critical complement C3 protein ($C3^{-/-}$) (Fig. 1B). Mice lacking C3 are unable to form MAC and induce cell lysis that results from any of the triggering pathways (Fig. 1A) (Sorbara et al., 2018). $C3^{-/-}$ and WT control C57Bl6/J mice were infected with the commonly-used mouse-adapted *H. pylori* strain PMSS1 (Arnold et al., 2011). After two weeks, total bacterial counts and gland colonization were evaluated in each of the two main stomach regions, antrum and corpus. As reported by others, *H. pylori* colonized the WT antrum to higher levels than the corpus (Keilberg, 2016; Fung et al., 2019). Some of this restriction was removed in the $C3^{-/-}$ mice. Total bacterial load in the $C3^{-/-}$ mice corpus was significantly elevated 100-fold compared to WT mice (Fig. 1C). Colonization in the important gastric sub-

region, the gastric glands, also showed that complement affected colonization most significantly in the corpus. The percent of infected glands and number of bacteria per gland each increased by 4-fold (Fig. 1D, 1E). These results suggest complement restricts *H. pylori* gastric colonization, especially in the corpus region.

2.4.2 *H. pylori* is sensitive to complement mediated elimination.

The above results show that removing complement from the mouse model elevates *H. pylori* numbers. Complement can both mediate direct cell lysis and modulate the immune response. *H. pylori* infections recruit immune cells slowly, and studies have shown that during infection period used here, there is limited immune cell recruitment to the site of *H. pylori* infection (Arnold, 2017), suggesting that a main role for complement may be to act via direct cell lysis. We therefore examined whether this *H. pylori* strain is vulnerable to complement-mediated cell lysis, as had been seen for other strains (Berstad et al., 2001), using exposure to active normal human serum (NHS). Active NHS eliminated *H. pylori* in a dose-dependent manner, while heat-treated inactive NHS was unable to kill *H. pylori* (Fig. 2A). Treatment with 10% NHS eliminated 60% of *H. pylori*, leaving 40% to survive, while treatment with 30% NHS eliminated 80% (Fig. 2A). To verify whether this elimination was complement-activation dependent, we treated the NHS with EGTA, a compound that blocks all three complement activation pathways (Berstad, 2001; Des Prez, 1975; Strobel, 2018). EGTA-treated NHS was unable to eliminate *H. pylori*, consistent with the idea that the active component in NHS is complement (Fig. 2B).

To validate whether this finding is applicable to the murine model and fully complement dependent, we extracted mouse serum from *H. pylori*-infection-free WT and $C3^{-/-}$ mice, and examined *H. pylori* survival ratio in each type of mouse serum. WT mouse serum eliminated around 75% of bacteria, while $C3^{-/-}$ mouse serum was only able to eliminate about 10% of the *H. pylori*, leaving nearly 90% of the population to survive (Fig. 2C). These results strongly suggest that complement activation is a major cause of *H. pylori* elimination during serum treatment.

2.4.3 Complement mediated clearance depends on classical/lectin pathway activation.

Complement can be activated through three pathways, classical, lectin, or alternative. These pathways can be differentially re-activated in EGTA-treated sera by adding back either Ca^{2+}, to activate the classical/lectin pathways, or Mg^{2+}, to activate the alternative pathway. We used this differentially-activated serum to evaluate which pathway targeted *H. pylor*i. Ca^{2+} supplementation resulted in the killing of *H. pylori* to levels that were nearly identical to those in untreated NHS (Fig. 2B). In contrast, adding back Mg^{2+} did not fully restore the killing capacity of the NHS (Fig. 2B). These results suggest that *H. pylori* is mostly targeted by the classical/lectin pathway, in agreement with previous findings with different *H. pylori* strains (Berstad et al., 2001).

2.4.4 *H. pylori* is able to resist complement in a manner that depends on lactate

Given that complement acts in the stomach to decrease *H. pylori* numbers, we hypothesized the bacterium has developed anti-complement strategies for its persistent colonization development. We speculated that *H. pylori* might sense some aspect of the gastric milieu and activate complement resistance. Although there are many candidate

conditions that could be sensed, we first tested whether lactate may play a role in facilitating *H. pylori* complement resistance, since lactate has been linked to complement evasion in other microbes (Exley, 2005) and has recently emerged as important in *H. pylori* physiology (Iwatani, 2014; Keilberg, 2021; Machuca, 2017). We therefore grew *H. pylori* with supplementation of physiological concentrations of L-lactate (Belenguer, 2007; Kahlert et al., 2016), and exposed the bacteria to NHS. Strikingly, the bacterial survival rate was significantly elevated, from ~40% to ~ 80%, (Fig. 3A), a response that was robust even in higher level of NHS exposure (Fig. 3B). Likewise, such an effect was detected from mouse serum exposure, raising the survival from ~22% (Fig. 2C) to ~80% (Fig. 3C). Lactate protected *H. pylori* from the classical/lectin pathway, activated by Ca^{2+}, significantly raising the survival percent from ~40% to ~85%, but not the alternative pathway, activated by Mg^{2+}, which did not differ ± lactate (Fig. 3E). Finally, we determined that the lactate effect happened rapidly, requiring only 1 hour of lactate exposure before NHS treatment to protect 70% of bacteria from elimination (Fig. 3D), a number that was similar to long-term lactate exposure (Fig. 3A). We then confirmed that L-lactate is present in the mouse stomach, by isolated tissue and quantifying total L-lactate levels. In uninfected WT or C3-/- mice, L-lactate levels were nearly the same, at 5 μmol/gram tissue, in both corpus and antrum (Supplemental Fig. 1). After two weeks of *H. pylori* infection, the levels were elevated 2-3-fold in the antrum and ~ 1.5 fold in the corpus (Supplemental Figure 1). This result supports the idea that *H. pylori* can be protected by gastric L-lactate during infection, possibly more-so in the antrum than corpus. Overall, these results suggest that lactate

exposure occurs in vivo and allows *H. pylori* to rapidly adopt a state that is resistant to both human and mouse complement.

2.4.5 L-lactate mediated *H. pylori* complement resistance requires uptake.

The above results show that lactate exposure allows *H. pylori* to become complement resistant, so we next determined whether this response requires lactate uptake to the cytoplasm. *H. pylori* has two lactate permeases called LctP1 and LctP2 (HP0140 and HP0141) that have been shown to promote lactate uptake in a distinct *H. pylori* strain (Iwatani et al., 2014). The *H. pylori* lactate uptake pathway shares the basic same principles as those initially identified in *Bacillus subtilis* and *Shewanella oneidensis* (Chai, 2009; Pinchuk *et al.*, 2009). To evaluate if complement resistance requires uptake, we created *H. pylori* PMSS1 mutants that lack the genes coding for both lactate permeases (Δ*lctP*) (Fig. 3F). The mutation was confirmed by PCR and the mutants analyzed for their response to L-lactate. The Δ*lctP* mutant strain was not able to acquire a growth advantage from L-lactate, as compared to WT *H. pylori* (Supplemental Fig. 2), supporting that the LctP transporters function as predicted in lactate update (Iwatani et al., 2014). When treated with NHS, the Δ*lctP* strain had very low survival, of only ~8% (Fig. 3G), 5-fold lower than WT (Fig. 3A). Addition of L-lactate did not improve this strain's survival (Fig. 3G). These results suggest that the anti-complement process in *H. pylori* requires L-lactate uptake.

2.4.6 L-lactate prevents accumulation of C4b on the *H. pylori* surface.

We next asked what step of complement activation was prevented by the lactate-treatment. Because lactate mainly protected *H. pylori* from the classical/lectin but not alternative pathways, we focused on the proteins and steps that are found only in the former (Fig. 1A). C4 cleavage is the first downstream activity of the classical/lectin pathways activation, generating two fragments: the larger C4b fragment that covalently binds to the target cell surface, and the smaller peptide C4a that is released (Fig. 4A) (Law & Dodds, 1997). We thus tested how L-lactate affects C4 activation. *H. pylori* was co-incubated with NHS, and the amount of C4b formation monitored over time by western blotting. C4 was rapidly activated in the presence of *H. pylori*, as monitored by C4b production and a decrease in total C4 at the end of the incubation period (Fig. 4B). C4b accumulation, however, was not different with or without lactate in WT *H. pylori*, nor did it change between WT and Δ*lctP* strains (Fig. 4B). This outcome suggests that lactate does not prevent C4b formation, and therefore does not affect steps upstream of this event. After C4 cleavage, C4b deposits on the cell surface. We therefore examined the status of C4b deposition. WT *H. pylori* was treated as above, and then bacteria were pelleted to estimate bound-C4b using western blotting. In this case, there was much less stably bound C4b associated with lactate-grown *H. pylori* as compared to non-treated bacteria (Fig. 4C). Control experiments showed that similar amounts of *H. pylori* were pelleted in all samples (Supplemental Fig. 3). L-lactate was not able to block C4b binding to the surface of the Δ*lctP* mutant (Supplemental Fig. 4). A well-known complement resistance mechanism that acts at

the C4 step is the use of the host protein Factor I with cofactors, such as C4 binding protein (C4BP) (Ermert, 2019; Jarva, 2005; Merle, 2015). C4BP and Factor I result in cleavage of C4b to two alternative forms: the bound but inactive C4d fragment and the released C4c fragment (Fig. 4A). To determine whether this pathway was operating, we monitored C4c formation, recognized by our anti-C4 antibody (Fig. 4B). C4c was nearly undetectable in either lactate-grown or non-lactate-grown WT and Δ*lctP H. pylori* (Fig. 4B), suggesting Factor I and C4BP were not acting. Considering that the Factor I-cofactor mechanism is the only known host regulatory mechanism that acts on C4 (Ermert, 2019; Merle, 2015), our findings suggest that a different resistance mechanism is taking place, possibly one that is bacterial-intrinsic. Overall, these results are consistent with a model in which lactate uptake results in a bacterial response that prevents the accumulation of C4b on the bacterial surface; blocking this step prevents the formation of the MAC and subsequent cell lysis.

2.4.7 Complement resistance is critical for stomach colonization

Our above results predict that *H. pylori* that does not mount a complement resistance response would have a severe colonization defect. We thus determined the performance of the *ΔlctP* mutant in the stomach by orally infecting WT mice. Remarkably, *ΔlctP* was nearly unable to colonize the stomach (Fig. 5), a phenotype that was confirmed in another commonly used WT mouse model, C57BL/6N (6N) mice (Supplemental Fig. 4) (Howitt, 2011; Keilberg, 2016). Thus, we concluded that lactate uptake promotes *H. pylori* infection.

The defect of the Δ*lctP* strains could be due to either loss of lactate-dependent growth or complement resistance. To differentiate these two possibilities, we conducted *ΔlctP* mutant infection in $C3^{-/-}$ mice. We discovered that the CFUs of the *ΔlctP* mutant were significantly elevated in the corpus, compared to those in WT mice. Indeed, the levels of Δ*lctP* mutants in the corpus of $C3^{-/-}$ mice were not different from those in the WT mouse corpus (Fig. 5). We also examined whether there were possible microbiota differences between WT and $C3^{-/-}$ mice that may have accounted for colonization differences. WT and $C3^{-/-}$ mice were cohoused to allow microbiota mixing during infection and for four weeks prior. WT *H. pylori* colonization exhibited no statistically significant differences between cohoused (Fig. 5) and non-cohoused WT or $C3^{-/-}$ mice (Fig. 1A), indicating no substantial influence of the resident microbiota. Taken together, these data support the idea that L-lactate-uptake has a major role to protect *H. pylori* from host complement.

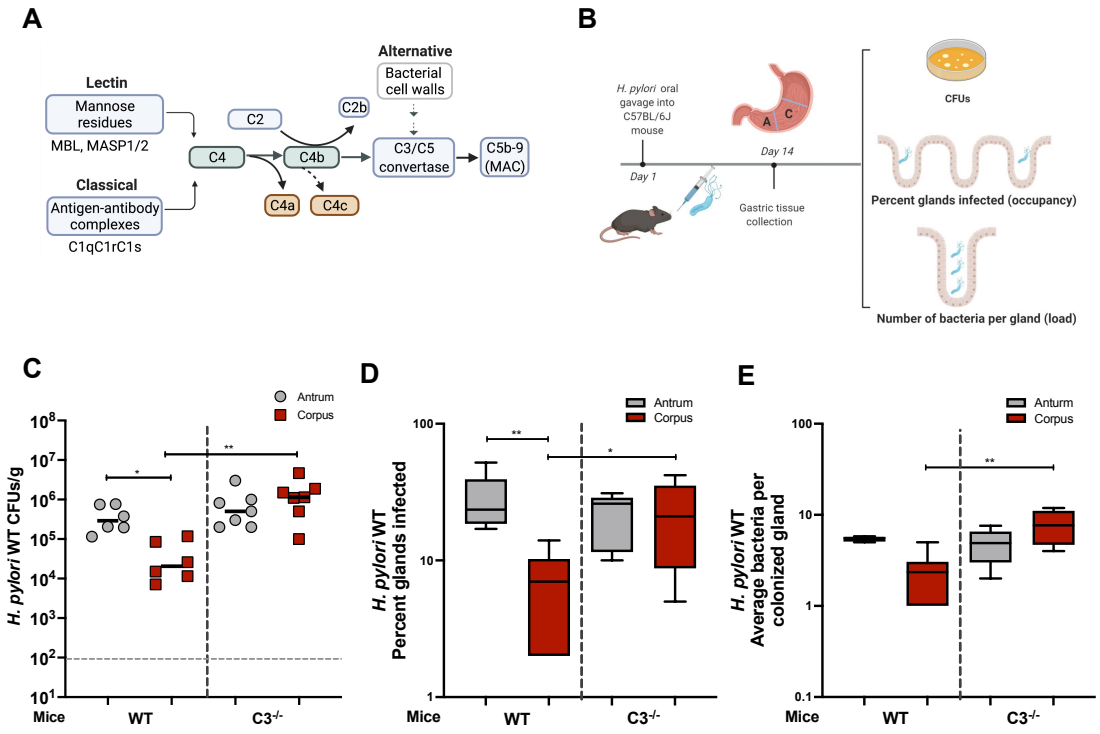


Figure 2.1 Complement plays vital role in restricting *H. pylori* gastric colonization.

(A) Schematic diagram of complement pathways activation. (B) Schematic illustration of mouse infection analysis. C57Bl6/J WT or $C3^{-/-}$ mice were infected by oral gavage with GFP^{+} *H. pylori* PMSS1 for two weeks. Stomachs were isolated from infected mice and separated into corpus and antrum pieces and processed to obtain the total bacterial numbers (colony forming units, CFUs) by plating, to enumerate bacteria in glands using microscopy. (C) Total bacterial number from gastric antrum (gray) and corpus (red) of infected WT or $C3^{-/-}$ mice normalized to tissue weight (CFUs/g). Gray dotted line, limit of detection. (D) Percentage of glands occupied by *H. pylori* in the gastric antrum (gray) and corpus (red) of infected WT or $C3^{-/-}$ mice, determined by analyzing 100 randomly selected isolated glands. (E) Number of *H. pylori* per colonized gland in

the gastric antrum (gray) and corpus (red) of infected WT or $C3^{-/-}$ mice. For all panels, black bars represent mean of samples; error bars (E, F) represent standard deviation (SD). The p-values were obtained by one-way ANOVA (Tukey's test). The significance is indicated as * ($P < 0.05$); ** ($P < 0.01$).

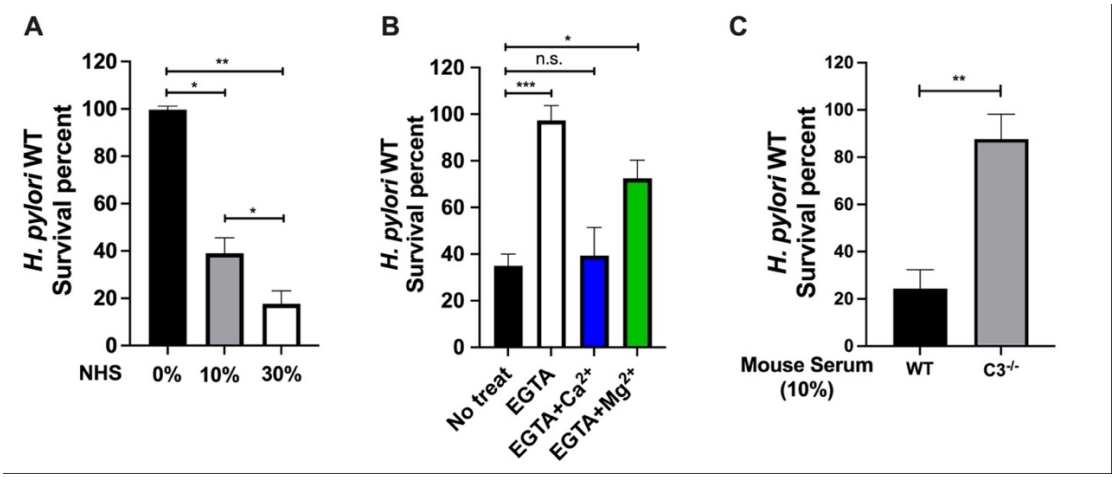


Figure 2.2 *H. pylori* is sensitive to complement mediated elimination

Overnight cultures of *H. pylori* PMSS1 WT were treated by inactive and active NHS or mouse serum at the indicated final percentages for one hour, and then viable bacterial CFUs were determined by plating. The survival percent was determined by comparing the number of CFUs from active serum divided by the CFUs from heat-inactivated serum treatment. (A) Comparison of different final percentages of NHS. (B) *H. pylori* cultures were treated by 10% EGTA-conditioned NHS to block activation of all three complement pathways. Mg^{2+} or Ca^{2+} were individually added back to the culture to

resume either the classical/lectin or alternative pathway activation. (C) *H. pylori* cultures were treated by 10% final percentage of extracted mouse serum from C57Bl6/J WT or $C3^{-/-}$ mice. In all panels, results were presented as survival percent ± standard deviation (SD), and were derived from triplicate biological samples with a triplicate of each given sample. The p-values were obtained with a two-tail student *t*-test. The significance was indicated as * ($P<0.05$), ** ($P<0.01$), and *** ($P<0.001$).

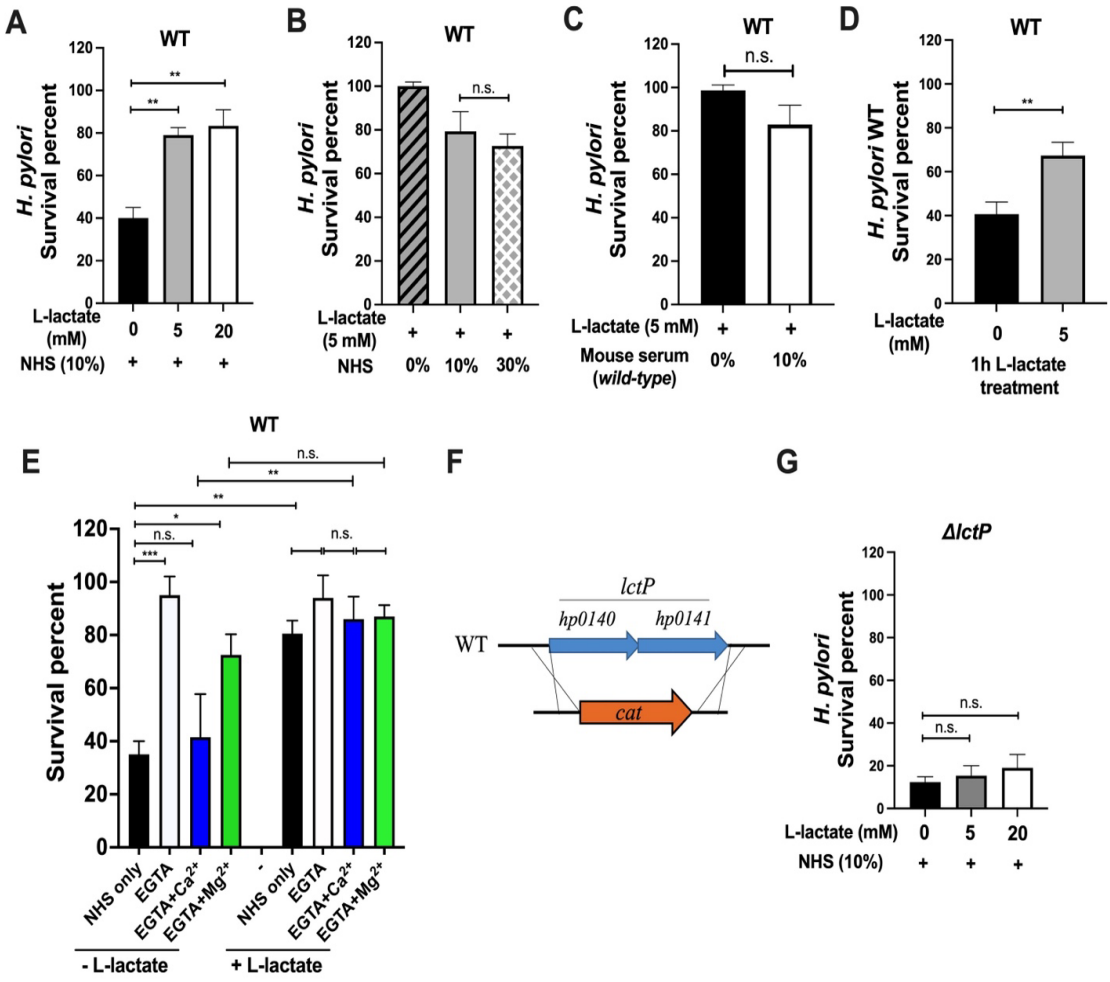


Figure 2.3 L-lactate protects *H. pylori* from complement mediated elimination

Overnight cultures of *H. pylori* PMSS1 WT or Δ*lctP* mutant with or without L-lactate supplementation overnight or for one hour, as indicated, were treated by inactive and active serum for one hour, and then viable bacterial CFUs were determined by plating. The survival percent was determined as described in Fig. 2. In all panels, results were presented as survival percent ± standard deviation (SD), and were derived from triplicate biological samples with a triplicate of each given sample. The p-values were obtained with a two-tail student *t*-test. The significance is indicated as * (P<0.05), **

(P<0.01), *** (P<0.001), or n.s. (not significant). (A-B). WT *H. pylori* treated with NHS. (C). WT *H. pylori* treated with C57Bl6/J mouse serum. (D) WT *H. pylori* treated with L-lactate for 1 hour before exposed to NHS. (E) WT *H. pylori* treated with NHS plus EGTA, or EGTA plus Mg^{2+} or Ca^{2+} as in Fig. 2. Survival ratio in the absence of L-lactate is identical to Fig. 2 (F) Schematic diagram of the mutation of the two *H. pylori lctP* orthologous genes. *cat*: chloramphenicol resistant gene. (G) Overnight cultures of *H. pylori* PMSS1 *ΔlctP* mutant grown with the indicated concentration of L-lactate were treated by inactive and active 10% NHS.

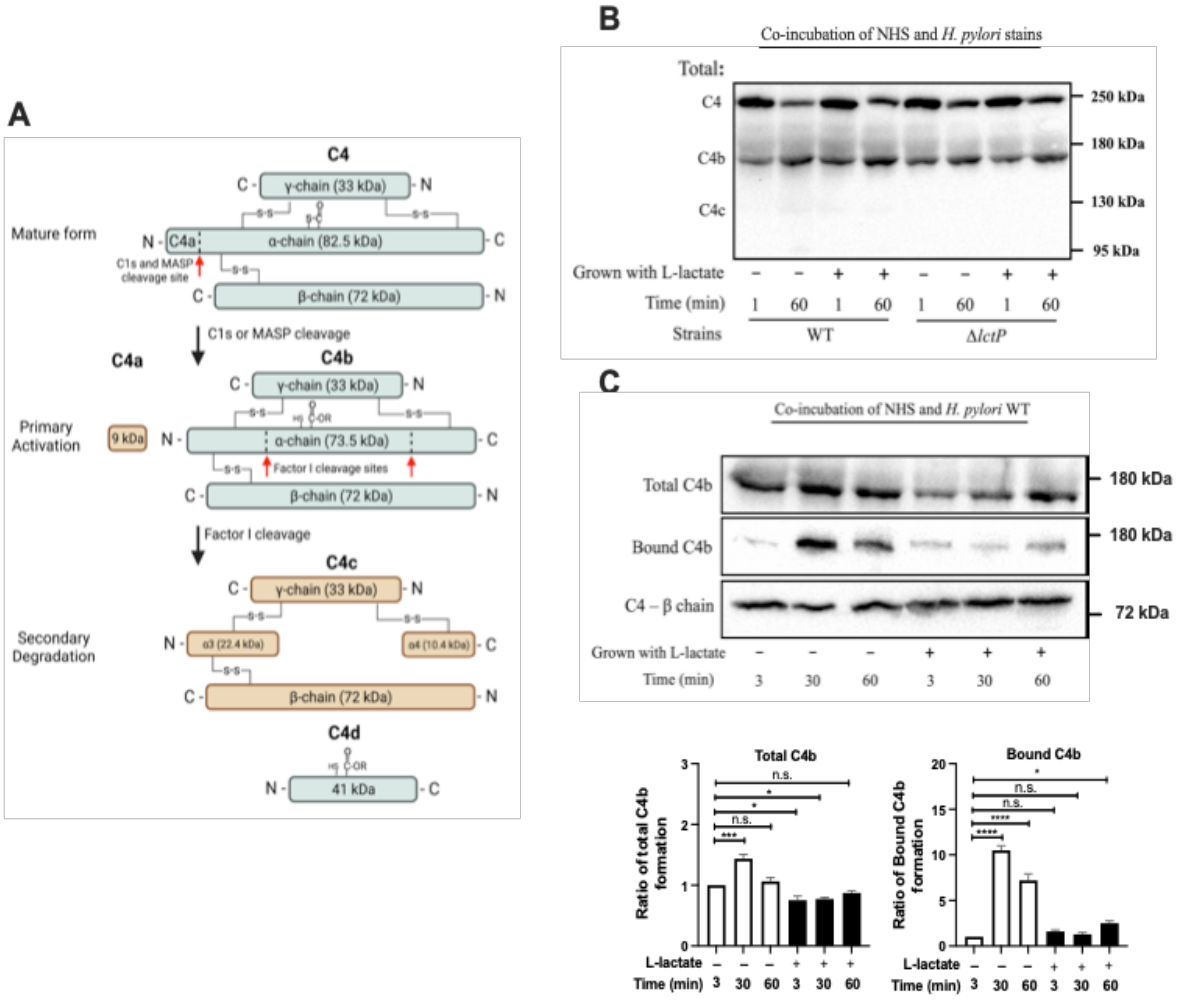


Figure 2.4 L-lactate mediates destabilization of C4b binding to *H. pylori*

(A) Schematic diagram of C4 primary activation and secondary degradation, adapted from (Law & Dodds 1997). (B) Western blot of C4 protein and products after activation in the presence of WT and Δ*lctP H. pylori* over 60 min, using non-reducing gels. Positions of C4, C4b, and C4c are shown at the left, and molecular weight is indicated on the right. (C) *H. pylori* PMSS1 WT was grown ± L-lactate and then treated by 10% NHS for the indicated time periods. Each sample was split into three: (1) treated by sample buffer only (total C4b); (2) treated by sample buffer plus the reducing agent

beta-mercaptoethanol (BME) (C4 β-chain); (3) centrifuged at 3,000g for 3 min before adding sample buffer (without BME) (bound C4b). All protein samples were run on 5-7% SDS PAGE and blotted with anti-C4 antibody. Representative immunoblots are shown from three independent experiments. For quantification purpose, bands were quantified by Bio-Rad Image Lab software. The data were combined from the three independent experiments (represented as mean ± SD). The p-values were obtained by one-way ANOVA (Tukey's test). The significance is indicated as * (P<0.05), *** (P<0.001), and **** (P<0.0001), or n.s. (not significant).

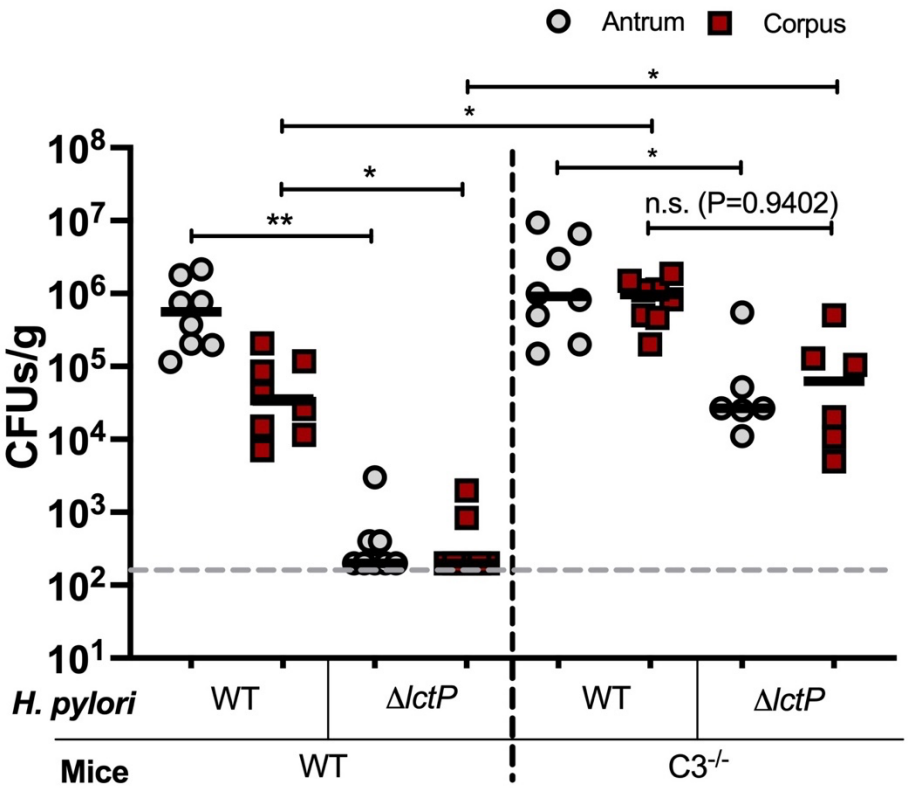


Figure 2.5 Loss of *lctP* renders *H. pylori* colonization deficient in a manner that is recovered by loss of complement

Groups of C57BL/6J WT and isogenic $C3^{-/-}$ mice were inoculated with the *H. pylori* PMSS1 WT or *ΔlctP* strains. All the WT and $C3^{-/-}$ mice were co-housed for 4-weeks before the infection and remained co-housed for the whole experimental period. for the entire experimental period. Bacterial CFUs were determined by plating. Each point represents one mouse sample, with bars representing the mean. Gray dotted line represents the limit of detection. The p-values were obtained by two-way ANOVA (Tukey's test). The significance was indicated as * ($P<0.05$), ** ($P<0.01$), and n.s. (no significance).

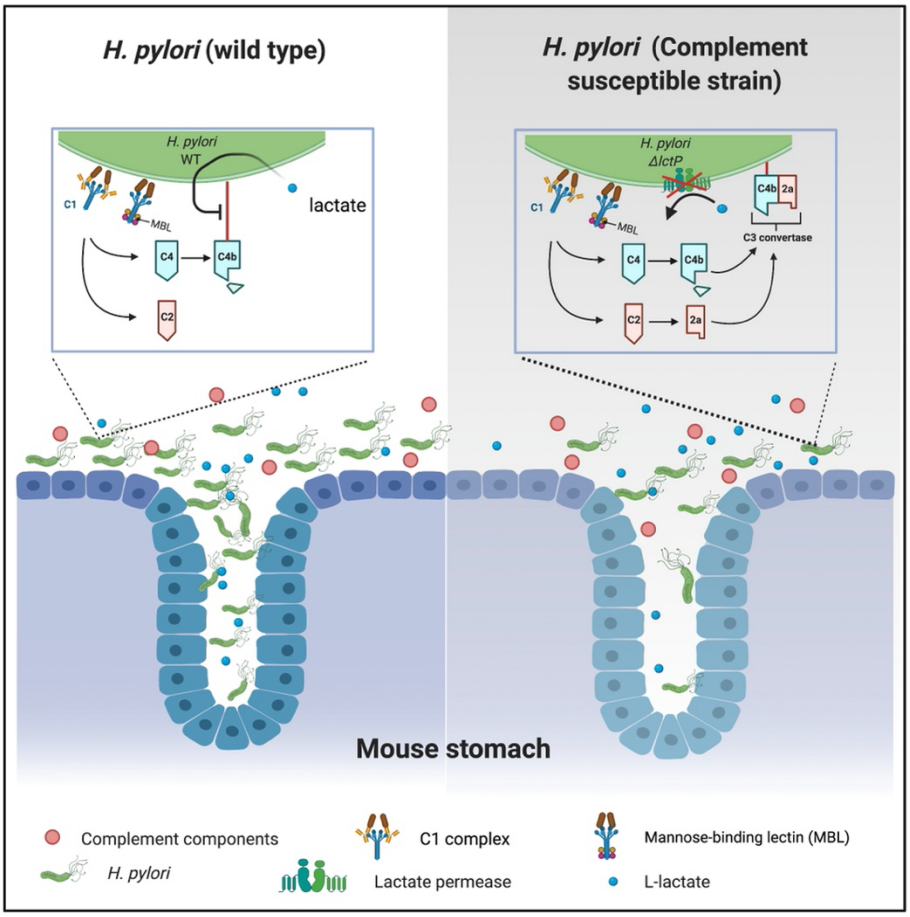
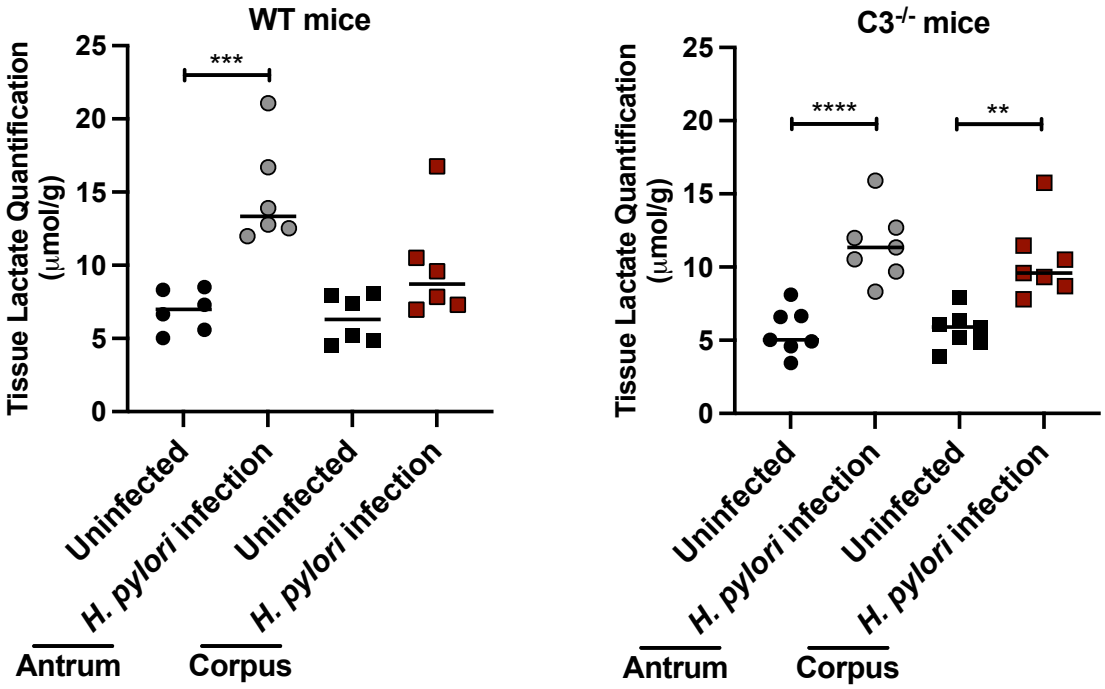


Figure 2.6 Proposed model for L-lactate promotion of *H. pylori* gastric colonization

The mammalian host has sophisticated and diverse mechanisms to prevent infection. One such mechanism is the complement system. Complement plays an important role to limit *Helicobacter pylori* gastric infection. *H. pylori* in turn, has evolved a strategy to diminish this detrimental impact via uptake of the host molecule L-lactate. The mechanism of L-lactate mediated resistance is distinct from that used by other bacteria, as it blocks surface deposition of the complement component C4b.

Strain	Genotype or description	Reference and/or source(s)
PMSS1	WT strain	Arnold et al., 2011
PMSS1 *ΔlctP*	*Δhp0140-0141::cat*	This study (KO1710)
PMSS1 pTM115	GFP^{+} PMSS1 WT	This study (KO1568)
PMSS1 *ΔlctP* pTM115	GFP^{+} PMSS1 *ΔlctP*	This study (KO1711)

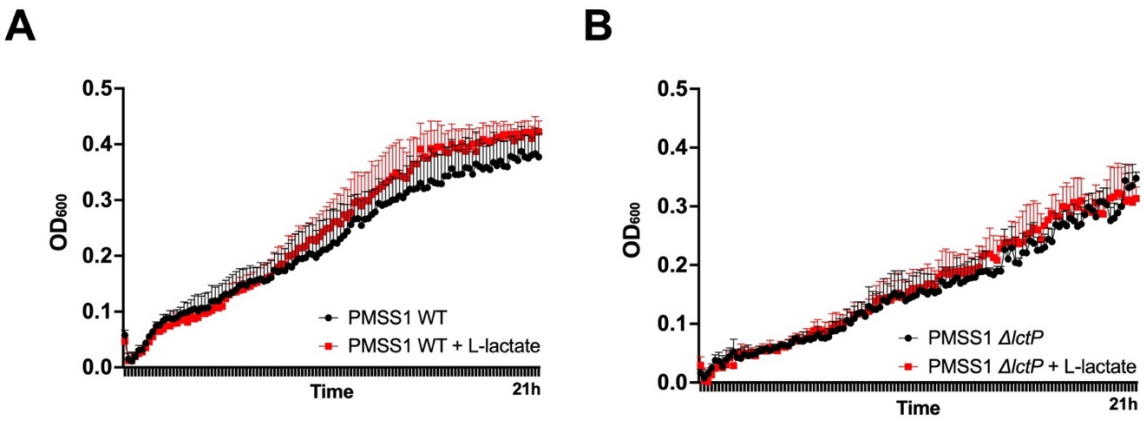
Supplemental Table 2.1 Strains used for this study



Supplemental Figure 2.1 L-lactate is overproduced during *H. pylori* infection

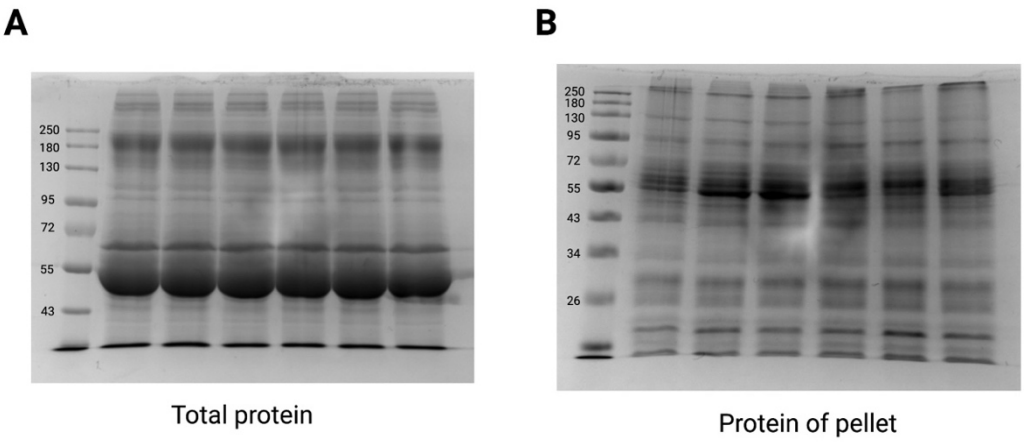
C57Bl6/J WT or C3$^{-/-}$ mice were infected by oral gavage with GFP^{+} *H. pylori* PMSS1 for two weeks. Stomachs were isolated from infected mice and separated into corpus and antrum pieces. A piece of tissue from each region was homogenized for

plating to obtain the total colony forming units (CFUs) as done in Fig. 1B and for lactate concentration measurement with lactate colorimetry assay kit (Biovision). Each point represents one mouse sample, with bars representing the mean. Statistical analyses were performed using one-way ANOVA with Tukey post hoc test, indicated as ** ($P < 0.01$), *** ($P < 0.001$), and **** ($P < 0.0001$).



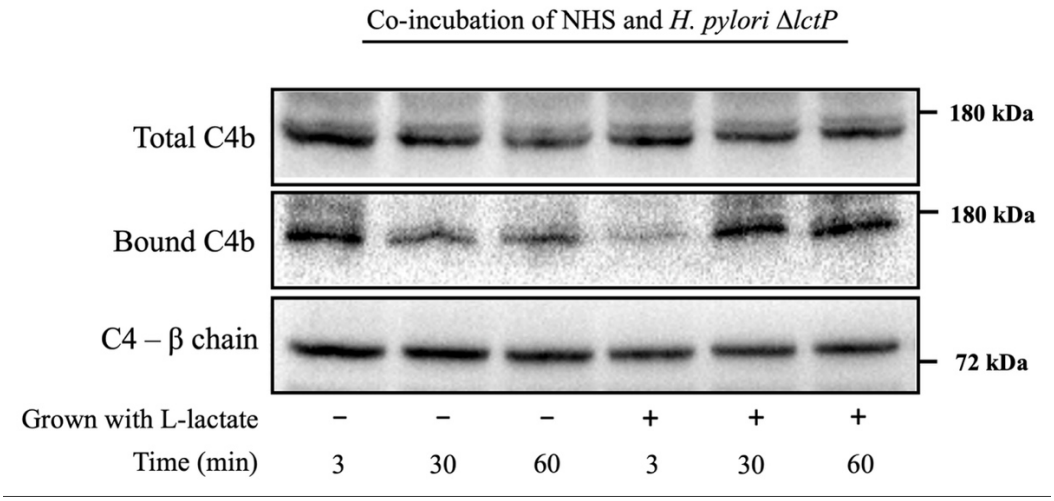
Supplemental Figure 2.2 LctP is required for growth advantage acquirement

Growth of *H. pylori* PMSS1 wild type (A), the *ΔlctP* mutant (B) were cultured in BB10 medium with (red dots) or without (black dots) L-lactate supplementation (10 mM of final concentration). The cell growth was monitored by measuring optical density at 600 nm (OD_{600}). The data points and error represent the mean ± standard deviation (SD) of three independent experiments.



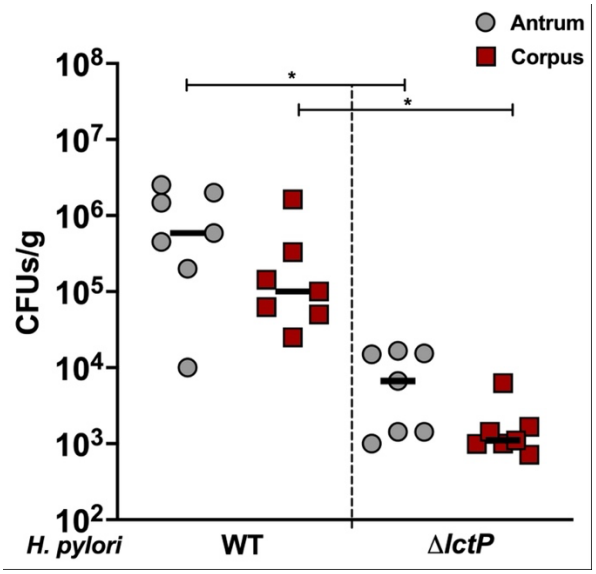
Supplemental Figure 2.3 equal amount of pelleted *H. pylori* was loaded for examination

H. pylori PMSS1 WT was grown ± L-lactate and then treated by 10% NHS for the indicated time periods. Each sample was split, (A) with one sample treated by sample buffer only (total protein), or (B) centrifuged at 3,000g for 3 min before adding sample buffer without BME to equal amounts as in (A) (protein of pellet). Equal amounts of protein samples were loaded as for Fig. 4D. Protein samples were run on 7-10% SDS PAGE and stained by Coomassie Blue.



Supplemental Figure 2.4 Bound C4b was stably bound to Δ*lctP* surface

H. pylori PMSS1 *ΔlctP* mutant was grown ± L-lactate and then treated by 10% NHS for the indicated time periods. Protein samples were prepared and blotted as for WT *H. pylori* showed in Fig. 4D.



Supplemental Figure 2.5 Δ*lctP* mutant is deficient to colonize C57BL/6N mice

C57Bl6/N mice were infected by oral gavage with GFP$^+$ *H. pylori* PMSS1 WT or *ΔlctP* mutant for two weeks. Stomachs were isolated from infected mice and separated into corpus and antrum pieces. A piece tissue of each region was homogenized and plated to obtain the total colony forming units (CFUs). Total bacterial number from antrum (gray) and corpus (red) of infected C57Bl6/N mice normalized to tissue weight (CFUs/g). Statistical analyses were performed using one-way ANOVA with Tukey post hoc test, indicated as * ($P < 0.05$).

2.5 Discussion

We report here that the complement system plays an important role in GI tract defense by mediating bacteriolytic activity during early colonization. We also uncovered that this detrimental effect was diminished by lactate uptake-mediated destabilization of C4b at the *H. pylori* surface. By infecting complement-deficient mice with lactate-uptake deficient *H. pylori* strain, we further demonstrate that a major role of lactate utilization is to promote complement resistance (Fig. 6). Because complement plays both immune-activation and immune-regulatory functions, our work suggests the idea that pathogens may have optimized particular methods of complement inactivation that protect themselves but also allows some actions of complement to proceed.

Previous work had identified that there were some complement components in the stomach, intestine, and colon, but it was not clear how these contributed to host defense. Indeed, the prevailing view was that many of these complement proteins were

found in dysregulated or chronic infection situations such as Crohn's disease, ulcerative colitis, or chronic gastritis (Halstensen, 1990, 1992; Berstad, 1997), but with an unknown function in acute infection. Complement proteins were not detected in the GI tracks of healthy hosts (Kopp, 2015). In the case of gastric infection, previous work had found that complement depletion decreased the severity of *Helicobacter felis* induced gastritis in an IL-10 deficient mouse model (Ismail, 2003). However, it was not clear whether complement played a role in wild-type animals during early infection. Our work clearly shows that eliminating complement from wild-type background, by loss of C3, increases the *H. pylori* load.

This effect of loss of complement occurred in both the antrum and corpus, but was greater in the latter. This bacterial load difference could be due to higher complement levels in the corpus, or alternatively, higher complement resistance in the antrum. Interestingly, our work showed that *H. pylori* infection elevated a L-lactate levels more in the antrum than the corpus. This finding suggests possibly that *H. pylori* is more complement resistant in the antrum, due to high lactate levels, so does not show a great effect of C3 loss. Complement lowers bacterial numbers by several mechanisms, including direct lysis and opsonization. We proposed that cell lysis dominates in vivo because there are few immune cells in *H. pylori* early infection (Arnold, 2017). Our finding that *H. pylori* blocks C4b deposition supports this proposal, because a blockade at this step would also prevent creation of the C3a and C5a products, which are immune cell chemoattractants.

Our work shows that *H. pylori* has a previously undescribed way of blocking complement. We found lactate-exposed *H. pylori* had much reduced bacterial-associated C4b, which is not due to Factor I cleavage. A bacterial-intrinsic C4 removal resistance mechanism has not been reported. Additional studies will be needed to determine how *H. pylori* decreases deposited C4b. C4b has a labile internal thioester bond that reacts broadly with amino and hydroxyl groups (Dodds, 1996), so it seems unlikely that lactate-treatment removes the target of C4b. Instead, it seems more likely that *H. pylori* removes the C4b, possibly by rapidly shedding the target molecules or employing some type of protease. This new complement resistance mechanism is in addition to several other *H. pylori* ones that utilize host proteins or carbohydrates. For example, *H. pylori* employs host CD59 to block MAC formation (Rautemaa, 2001). *H. pylori* virulence factor SabA is able to bind sialic acid, which facilities blocking alternative pathway activation (Unemo, 2005; Blaum, 2015). It indeed seems that *H. pylori* has complex and robust complement resistance mechanisms to cope with this host defense system, emphasizing the importance of complement in *H. pylori* biology.

We speculate that *H. pylori* blocks this particular step for a reason. Allowing cleavage of C4b would also form C4a. So, one idea is that the microbe gains a benefit from C4a. C4a is an agonist of protease-activated receptor (PAR)1 and PAR4 (Wang, 2017), which has been previously demonstrated to induce intestinal epithelial apoptosis in a caspase-3-dependent manner (Chin, 2003). Apoptosis of gastric epithelial cells is a hallmark of *H. pylori* infection, possibly as a way to acquire nutrients (Cover, 2003; Kohda, 1999). Apoptosis induction, as a way to acquire nutrients, occurs in other

pathogens. For example, *Salmonella* induces caspase-3/7-dependent epithelial apoptosis in the intestine to acquire important nutrients, such as pyruvate, that promote its growth (Anderson et al., 2021). Other evidence suggests genes for complement proteins may be upregulated during *H. pylori* infection. Guillemin an colleagues found that the complement component C4 coding gene C4B, is upregulated upon *H. pylori* infection of gastric epithelial cells (Guillemin, 2002). This response may indicate that *H. pylori* has evolved to manipulate the complement system to its benefit, e.g. to help promote apoptosis.

Another finding reported in this work is the expansion of our knowledge of how *H. pylori* utilizes lactate. Lactate utilization has been reported to support microbial infection in various ways, including both as a growth substrate and for other reasons. *N. meningitidis*, for example, utilizes lactate to extend its bloodstream infection period by promoting complement resistance (Exley, 2005, 2007). Our study demonstrated that *H. pylori* also uses lactate for complement resistance. However, key differences exist between these two bacterial systems. *N. meningitidis* utilizes lactate to evade alternative pathway activation by stimulating the sialic acid biosynthesis pathway (Exley, 2005), while in *H. pylori*, L-lactate mediates classical/lectin pathway defense by promoting the removal of C4b. One interesting aspect of *H. pylori* is that it has duplicated *lctP* genes (*lctP1* and *lctP2*) on the *H. pylori* genome, a configuration that is atypical (Iwatani, 2014; Exley, 2005). Overall, these findings indicate a divergent bacterial environmental adaptation from a common nutrient acquisition origin.

The mechanism that connects lactate to the anti-complement response in *H. pylori* is not yet clear. Results presented here show that *H. pylori* promotes lactate production in the stomach. The source of lactate is not yet known, but many fast growing cells, such as epithelial cells, glandular stem cells, or even activated phagocytic cells are able to produce lactate and potentially impact local lactate concentration (Jiang, 2017; Le, 2010; Gu et al., 2016; Schell et al., 2017; Nolt et al., 2018; Remero-Garcia et al., 2016). The combination of regional L-lactate accumulation together with complement restriction may dynamically influence *H. pylori* gastric localization. One idea is that *H. pylori* survives and colonizes the local niches where lactate is relatively abundant. An important observation that supports this idea is that *H. pylori* colonizes the progenitor and stem cells compartments and stimulates $Lgr5^{+}$ stem cells proliferation (Sigal et al., 2015). Stem cells feature a high glycolytic rate compared to more differentiated cells (Gu et al., 2016), that likely produce more lactate and serve as a source for *H. pylori*. Our work suggests that L-lactate may function as a signaling molecule, acting to activate transcriptional or translational factors to regulate the expression of *H. pylori* genes. Other microbes have lactate responsive transcription factors, including *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans* (Aguilera, 2008; Gao et al., 2012; Ballou et al. 2016). In *C. albicans*, lactate binds to the lactate sensor Gpr1 that further activates the transcription factor Crz1 to regulate membrane integrity (Ballou et al, 2016). *H. pylori* has no known homologs of lactate sensing transcription factors, so future studies will need to work out this pathway.

More than half of the world's population is infected with *H. pylori*, which causes a series of gastric diseases (Hooi et al., 2017). Treatment relies on "triple-therapy", consisting of two antibiotics and a proton pump inhibitor (Chey, 2017; Segura, 1997). There are increasing reports about antibiotic resistance, such that *H. pylori* has been placed on the WHO's global priority list of antibiotic resistant organisms (Ghotaslou, 2015; Savoldi, 2018; Tacconelli, 2017). Our study strongly indicates that L-lactate utilization is tightly intertwined with *H. pylori* colonization, beyond sole use as a carbon source (Iwatani, 2014). Thus, we envision therapeutics that might block lactate uptake, and in turn result in robust complement-mediated elimination of *H. pylori*.

2.6 Methods

2.6.1 Bacterial strains and growth condition

H. pylori PMSS1 (Arnold et al., 2011) and isogenic mutant were used for these studies (Table 1). The isogenic *ΔlctP* mutant (KO1710) was constructed using a PCR-based method as described previously (Chalker et al., 2001). To delete the lactate permease coding genes, the coding region of both *hp0140* and *hp0141* were entirely replaced by a chloramphenicol resistance cassette (*cat*) (Fig. 3F), selected by chloramphenicol (25 μg/ml) and the mutants verified by PCR amplification. For mouse model infections, GFP^+ PMSS1 WT and *ΔlctP* strains were constructed by natural transformation with plasmid pTM115 (Keilberg et al., 2016) to create PMSS1 pTM115

(KO1568) and PMSS1 *ΔlctP* pTM115 (KO1711). For the transformation, plasmid isolated from *H. pylori* strain SS1 pTM115 was used with selection CHBA plates containing 15 µg/ml kanamycin.

For solid media, *H. pylori* strains were grown on Colombia Horse Blood Agar containing: 5% defibrinated horse blood (Hemostat Laboratories, Dixon, CA), 0.2% β-cyclodextrin, 10 µg/ml vancomycin, 5 µg/ml cefsulodin, 2.5 U/ml polymyxin B, 5 µg/ml trimethoprim, and 8 µg/ml amphotericin B (CHBA) (all chemicals are from Thermo Fisher or Gold Biotech). For antibiotic resistance marker selection, bacterial media was additionally supplemented with 25 µg/ml chloramphenicol or 15µg/ml kanamycin. For liquid media, *H. pylori* cultures were grown in brucella broth (BB) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (BB10) (Life Technologies). *H. pylori* were grown under microaerobic conditions of 10% CO_2, 5% O_2 and 85% N_2 at 37°C.

2.6.2 *H. pylori* growth assays

CHBA-maintained *H. pylori* strains were resuspended in BB10 media at an initial optical density 600nm (OD_{600}) of 0.1. Sodium L-lactate (Sigma) was added to a final concentration of 10 mM. 100 µl of this bacterial culture were placed in wells of 96-well plates and grown with 200 RPM constant shaking , at 37°C, 5% O2 and 10% CO2 atmosphere, using a FLUOstar microplate reader (BMG Labtech). OD_{600} was measured using the microplate reader during growth.

2.6.3 Serum resistance assays

H. pylori infection-free normal human serum (NHS, Sigma) was used for *H. pylori* serum sensitivity tests. The NHS was either left untreated (active) or heat-inactivated by heating in a water bath for 60 min at 56 °C. For serum-mediated killing assays, overnight bacterial cultures were diluted to OD_{600} of 0.1 in fresh matched medium (BB with or without lactate), and then NHS (active or inactive) was added to the final volume percentage of 10% or 30%. In some cases, complement pathways were queried by addition of 1mmol/L EGTA (Sigma), 1.2 mmol/L $CaCl_2$ or 1.2 mmol/L $MgCl_2$ (all final concentrations) (Berstad et al., 2001). Samples were incubated for 1 hour under microaerobic conditions with shaking. After the desired time, samples were serially diluted and plated on CHBA, and incubated for five days to allow colony formation. Statistical comparisons were made using a two-tail Student's T-test. Differences in *P-value* of < 0.05 were considered significant.

2.6.4 Mouse infection

The University of California, Santa Cruz Institutional Animal Care and Use Committee approved all animal protocols and experiments (Protocol OTTEK1804 and OTTEK2014). Female C57BL/6N mice (Helicobacter-free, Charles River Laboratories), C57BL/6J mice (Helicobacter-free, The Jackson Laboratory) and B6;129S4-C3<tm1Crr>/J mice ($C3^{-/-}$) (Helicobacter-free, The Jackson Laboratory) were housed at the animal facility of the University of California Santa Cruz.

Mice were between 7 to 9 weeks old at the time of *H. pylori* infection. For some infections, $C3^{-/-}$ mice were co-housed with their wild-type parental C57BL/6J mice for

4-weeks before the infection and remained co-housed for the whole experimental period. All animals were orally infected via a 20-gauge 1.5-in. feeding needle (Popper) with 100 µl containing ~10^8 GFP^+ *H. pylori* strains. These strains were prepared by liquid growth to late-exponential phase in BB10 medium. After the infection period, the animals were sacrificed via CO_2 narcosis, the stomach dissected, opened along the lesser curvature, and the forestomach region removed and discarded. The remaining tissue was divided into three parts—the corpus, the transition zone, and the antrum, as described previously using difference in tissue coloration as a marker (Rolig et al., 2012). The transition zone was discarded and not included in experimental analysis. Each region was then divided into two pieces with 0.3-0.5 cm^2 of each, one piece for total bacterial number counting and lactate concentration measurement, and the other for gland isolation. For total bacterial number counting, the tissue was weighed, homogenized using the Bullet Blender (Next Advance) with 1.0-mm zirconium silicate beads, diluted and plated to determine the number of CFUs/gram of stomach tissue on CHBA with the addition of 20 μg/ml bacitracin, 10 μg/ml nalidixic acid, and 15 μg/ml kanamycin. For lactate concentration measurement, the homogenized tissue lysates were then performed by using Lactate Colorimetry Assay Kit (Biovision, Milpitas, CA) according to manufacturer's instruction. For gland isolation, glands were isolated using a protocol described in Keilberg et al., 2016, and kept on ice until examined.

2.6.5 Mouse blood collection

Housed *Helicobacter*-free C57BL/6J mice were used for blood serum extraction. CO_2 narcosis was performed to euthanize the mice. Cardiac puncture was carried out using

a 3 cc syringe with a 22 gauge x 1" needle. The needle was advanced in the notch to the left of the mouse's xiphoid, into the chest, and heart. Once the needle was in the heart, the plunger was withdrawn to syringe generate slight back pressure to allow blood flow into the syringe. Each mouse enabled ~800 cc blood collection. The collected blood was allowed to clot for 1 hour on ice without any coagulant inhibitors. The clotted material was removed by centrifugation at 3000 rpm for 15 min. The top serum was collected and stored at -80°C for future analysis.

2.6.6 Gland analysis and microscopy

Isolated glands were labeled with 10 μg/ml Hoechst DNA stain (Life Technologies). Ten microliters of labeled glands were placed on glass slides and visualized using a Nikon Eclipse E600 microscope with fluorescence filters for 4',6'-diamidino-2-phenylindole (DAPI) and GFP. For each time point of infection, 100 glands were imaged for the corpus and 100 for the antrum, and the number of intra-gland *H. pylori* was manually counted for each gland. Gland occupancy was calculated as the percentage of glands occupied per mouse. Gland load were calculated by averaging the number of bacteria in occupied glands per mouse. Statistical analyses were performed using one-way ANOVA with Tukey post hoc test. The significance indicated as * ($P < 0.05$); ** ($P < 0.01$).

2.6.7 Serum C4 activation assay

Overnight *H. pylori* PMSS1 WT and mutants were suspended into BB media containing ~10^7 bacteria. Active NHS was added to the bacterial culture and allowed to co-incubate for indicated time periods at 37°C. Reactions were stopped by adding

6x sample buffer (0.3M Tris-HCl, pH 6.8, 6 M glycerol, 10% w/v SDS, 9 mM bromophenol blue). Some protein samples were further treated with final 5% β-mercaptoethanol to cleave disulfide bonds. All samples were then incubated at 90°C for 10 min. C4 activation was analyzed by monitoring the formation of cleaved C4 portion, C4b, through western blot.

2.6.8 Western blot

Samples were separated on 5% or 7% (wt/vol) acrylamide SDS/PAGE gels, and then used for immunoblotting. For immunoblotting, gels were soaked in transfer buffer [48 mM Tris-base, 39 mM glycine, 1.3 mM SDS, 20% (vol/vol) methanol] for 10 min and then transferred to an immunoblot polyvinylidene fluoride (PDVF) membrane (Bio-rad) for 60 min at 25 V through Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad). The membrane was blocked for 1 h with blocking buffer (PBS with 5% milk plus 0.2% Tween-20) at room temperature. Primary antibody was added and incubated for 16-18 h at 4 °C using the 1:1000 dilution for Rabbit anti-human C4 antibody (mAb #60059) (Cell Signaling). After incubation, the membranes were washed and HRP-conjugated Goat anti-rabbit or Rabbit anti-Goat secondary antibody (Santa Cruz Biotechnology) added at 1:10,000. After incubation, the membranes were washed and treated with HRP substrates (SuperSignal West, Pico and hydrogen peroxide mixed at 1:1 ratio; Thermo Scientific). Blots were then visualized using a Chemidoc imaging system (Bio-Rad).

2.7 References

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CHAPTER 3

Helicobacter pylori utilizes a lactate-induced transcriptional program including the gene for phospholipase PldA to evade complement activation.

3.1 Abstract

The complement system plays a vital role in restricting gastrointestinal bacterial infection. To avoid elimination, *Helicobacter pylori* has developed the capability to resist complement, a response that is induced by L-lactate uptake. Here we identify the basis for this anti-complement mechanism. We found that L-lactate played a role as signaling molecule that mediated the upregulation of a small regulon, including the phospholipase A (PldA) coding gene. PldA was critical to prevent killing by complement activation. PldA significantly enhanced *H. pylori* complement tolerance by efficiently dissociating C4b on the *H. pylori* surface. Without PldA, *H. pylori* survival was impaired under complement exposure, and mutants were unable to colonize mouse stomachs. This work highlights a previously unknown function of phospholipases in complement resistance, and a new type of bacterial anti-complement mechanism. Our results suggest PldA may plays essential role in facilitating *H. pylori* gastric colonization through facilitating complement resistance.

3.2 Introduction

Many microbes can chronically inhabit the mucosal surfaces of the gastrointestinal (GI) tract, utilizing sophisticated strategies to overcome diverse host restrictions. It was recently appreciated that one such microbial strategy is to remodel

host metabolism to acquire particular nutrients. For example, in the intestine, *Salmonella* triggers host immune responses that lead to the production of molecules such as the carbon source lactate and the anaerobic electron acceptor tetrathionate, allowing for advantageous expansion (Rivera-Chávez et al., 2016; Gillis et al., 2018). It is not yet clear whether this ability is widespread among bacterial pathogens, or whether the acquired benefit is limited to growth promotion.

One pathogen that is well known for its ability to maintain chronic mucosal colonization is *Helicobacter pylori*. *H. pylori* is a gastric pathogen and a leading cause of gastritis, peptic ulcer disease, mucosa-associated lymphoid tissue (MALT), and gastric adenocarcinoma (Marshall et al., 1995; Uemura et al., 2001; Peek et al., 2002). In fact, gastric adenocarcinoma represents the fourth leading cause of cancer-associated death worldwide, killing about 783,000 people every year (Amieva et al, 2016). Individuals generally acquire *H. pylori* in childhood, often resulting in lifelong colonization. *H. pylori* grows and survives in vivo, but the nutrients that support the microbe are not well understood. It furthermore is not clear whether *H. pylori* manipulates the gastric environment to obtain nutrients. We therefore reasoned the study of *H. pylori in vivo* infection would enhance our understanding of bacterial virulence and colonization strategies.

Previous metabolomics studies, using gastric organoids, discovered that *H. pylori* preferentially utilizes carboxylic acids and amino acids (Keilberg et al., 2021). One of the most highly consumed metabolites was lactate. Additional evidence for lactate's importance comes from the study of *H. pylori* chemotaxis: *H. pylori* was

attracted to lactate, one of six known attractants for this microbe (Machuka, et al, 2017; Johnson et al, 2018). L-lactate is used by *H. pylori* to promote growth (Takahashi et al., 2007; Iwatani et al., 2014). It is an important carbon and energy source for various other pathogens that colonize diverse niches, including *Neisseria meningitidis*, *Neisseria gonorrhea*, *Haemophilus influenzae*, *Staphylococcus aureus*, and *Salmonella enterica* serovar *Typhimurium* (Gillis *et al.*, 2018; Jiang *et al.*, 2014). In addition to growth, lactate utilization has been found to be involved in the immune defense process in *H. pylori* . The complement system plays an important role in restricting *H. pylori* gastric colonization (Chapter 2, Figure 1). To confront such restriction, *H. pylori* has developed a strategy to resist classical/lectin complement pathway activation by taking up environmental L-lactate. L-lactate promoted *H. pylori*'s ability to dissociate surface bound protein C4b, the product of complement component C4 proteolytical activation. The mechanism behind this dissociation and how lactate stimulates it is not known.

In this study, we aimed to explore the lactate mediated anti-complement mechanism. We performed transcriptomic analyses to define the lactate response. We identified several significantly upregulated genes, including the gene for phospholipase A (*pldA*). Further characterization identified that PldA dissociates the complement C4b component from the *H. pylori* surface. Overall, our results reveal that *H. pylori* can utilize a host derived metabolite as a nutrient and a signaling molecule simultaneously. When acting as signaling molecule, lactate stimulates *pldA* expression to develop a complement resistant state that consists of dissociating C4b from the surface, a previously undescribed mechanism.

3.3 Results

3.3.1 Lactate uptake but not metabolism is needed during gastric colonization

Our results shown in Chapter 2 demonstrate that lactate uptake is required for *H. pylori* gastric colonization. Specifically, the *H. pylori* Δ*lctP* mutant has a significant defect in gastric colonization. We therefore wondered whether lactate metabolism is similarly needed, as a downstream activity of lactate uptake. We thus genetically deleted L-lactate dehydrogenase (L-LDH) coding genes (*hp0137-0139*) (Iwatani et al., 2014) and orally infected wild-type C57BL/6N (6N) mice with GFP$^+$ *H. pylori* PMSS1 WT, and *ΔL-ldh* mutant, respectively. The 6N mice model was chosen for these experiments because *H. pylori* has a balanced distribution between the antrum and corpus regions (Howitt et al., 2011; Keilberg et al., 2016), which is helpful to reveal potential divergent phenotypes between local regions. After two weeks infection, we found that *H. pylori* WT was recovered at 10^5 CFUs/g level in both the antrum and corpus regions of infected stomachs (Fig. 1). Compared to WT *H. pylori*, the *ΔlctP* mutant was significantly less able to colonize in both regions, about 100-fold less respectively (Fig. 1), suggesting that lactate uptake is important for *H. pylori* gastric colonization. Surprisingly, the population of *ΔL-ldh* mutant was similar to those of WT in both the antrum and corpus, and indeed were even a bit higher in the antrum (Fig. 1). This result suggests that the *ΔlctP* mutant defect is not due to lactate metabolism, and likely has more to do with the known susceptibility to complement.

3.3.2 Lactate metabolism is dispensable for lactate-mediated complement resistance

The WT level of *ΔL-ldh* mutant load during early stage gastric colonization suggested that this mutant may not be involved in the complement resistance process. We therefore examined *ΔL-ldh* mutant sensitivity under complement exposure, using normal human serum (NHS). Our previous work in Chapter 2 showed that complement is the major factor in NHS that kills *H. pylori*. 40% of WT *H. pylori* grown from non-lactate condition was survived after NHS exposure (Fig. 2A). In contrast, 75% of *ΔL-ldh* mutant was survived, significantly higher than WT and much higher than that of complement sensitive strain (*ΔlctP*), at about 10% survival ratio (Fig. 2B-C). The survival ratio of the *ΔL-ldh* mutant was further enhanced by the present L-lactate, the trend that was also different from the *ΔlctP* mutant (Fig. 2B). To confirm this observation, we tested two other independent isogenic *ΔL-ldh* mutants and found the identical outcomes (data not shown). The result suggests that L-lactate-mediated complement resistance does not require L-LDH, which drove us to hypothesize that complement resistance is independent of L-lactate metabolism.

To test this hypothesis, we supplemented *H. pylori* WT, *ΔlctP,* and *ΔL-ldh* strains with pyruvate, the product of lactate oxidation via L-LDH, and examined their NHS sensitivity. Pyruvate substantially enhanced the capacity of the WT and *ΔlctP* mutant to resist NHS even at the lowest given concentration (Fig. 2D, 2E). However, pyruvate did not promote the resistance of *ΔL-ldh* mutant regardless of the supplemental concentration (Fig. 2F), suggesting that pyruvate mediated complement resistance is L-LDH dependent. These findings are consistent with the idea that *ΔL-ldh*

mutant is highly NHS tolerant because the block of L-lactate oxidation elevates internal L-lactate, and forces the majority of the acquired L-lactate to participate in an unidentified pathway which is responsible for complement resistance. In contrast, the *ΔL-ldh* mutant cannot convert pyruvate to L-lactate, and thus this mutant has low internal L-lactate. In sum, these results suggest that L-lactate metabolism is dispensable in this process. Our observations suggest a model in which L-lactate is taken up by LctP and then either utilized by L-LDH for growth or shunted to an L-LDH-independent serum resistance pathway (Fig. 2G).

3.3.3 Transcriptomic profiling revealed several genes are under lactate regulation

The above results show that L-lactate metabolism is not required for complement resistance, indicating that L-lactate may play a role as signaling molecule to regulate complement resistant gene expression. Consistent with this idea, previous work showed the lactate-induced complement response occurs within an hour. We therefore applied transcriptomic analysis to gain insight into the genes that are under lactate regulation. For this experiment, we grew *H. pylori* WT to stationary phase (optical density stop increasing) and then treated physiological concentration of lactate for 1 hour, a treatment that creates a complement resistant state. Total mRNA was then collected with rRNA depleted, converted to cDNA and sent for sequencing. A total 10 to 20 million reads per bacterial sample were generated by RNA-seq. These reads were then mapped to the *H. pylori* PMSS1 complete reference genome and revealed a clear subset of genes that were differentially expressed in the presence of lactate (Fig. 3A).

This transcriptomics analysis showed that three genes were particularly up-regulated, with more than a 2-fold change of genes expression. These are phospholipase A (*pldA*), 50S ribosomal protein L21 (*rplU*), and glutamate racemase (*murI*) (Fig. 3B). No genes were down-regulated (Figure 3B). A hypothetical gene *HPPMSS1_00673* was 1.85-fold upregulated that is below the 2-fold threshold but differential expression $p < 0.05$, so that it was considered as a potential target as well for following tests.

3.3.4 Phospholipase A promotes *H. pylori* complement tolerance

To verify whether these lactate-induced genes are involved in complement resistance, we generated isogenic mutants lacking each gene and examined their susceptibility to NHS exposure with and without lactate supplementation. Without lactate, the *pldA* and *rplU* mutant showed similar survival percentage as WT of about 40% (Fig. 4), while the HPYLPMSS1_0673 showed a higher survival (Fig. 4), similar to that seen with the *l-ldh* mutant (Fig. 2). With lactate supplementation, the *rplU* mutant and WT both showed increased survival, suggesting that *rplU* does not play a role in complement survival. The HPYLPMSS1_0673 showed a modest increase in response to complement, suggesting that this gene was either not playing a role in complement resistance or under an indirect regulation by L-lactate (Fig. 4). In contrast, the *pldA* mutant survival ratio was not increased with lactate supplementation (Fig. 4). These results suggest that PldA is an important factor in the lactate-triggered complement resistance.

3.3.5 PldA promotes C4b destabilization from *H. pylori* surface

In our previous study, we had demonstrated that lactate treatment effected a particular step of complement activation: the deposition of C4b deposition on *H. pylori*'s surface. Specifically, lactate treated *H. pylori* had normal C4b activation but did not retain C4b on the surface as did untreated cells. We, therefore, questioned whether PldA might play a role in blocking C4b deposition on *H. pylori* surface. The *H. pylori ΔpldA* mutant was co-incubated with NHS, then bacteria were pelleted to estimate bound-C4b using western blotting. WT *H. pylori* was able to block C4b deposition in the presence of lactate, as seen before (Chapter 2, Figure 4C), but in contrast, the *ΔpldA* mutant was not capable of dissociating C4b from its surface regardless of whether lactate was supplemented (Fig. 5). This outcome suggests that PldA plays an important role for destabilizing C4b from *H. pylori* surface.

3.3.6 PldA mutant was moderately recovered in complement deficient mouse model

We then wondered whether *H. pylori* lacking *pldA* would have a colonization defect. To test this possibility, we determined the performance of the *ΔpldA* mutant in the mouse infection model. The *ΔpldA* strain was nearly unable to colonize the stomach (Fig. 6), similar as *ΔlctP* infection (Chapter 2, Figure 5). This outcome supports the idea that PldA is required for *H. pylori* gastric colonization, matching findings obtained with a different *H. pylori* strain and using different mice (Dorrell et al., 1999).

We next questioned whether the *ΔpldA* mutant would colonize better in complement deficient mice, as the *ΔlctP* strain did. To answer this question, we

conducted *ΔpldA* mutant infection in $C3^{-/-}$ mice, as we did for the *ΔlctP* mutant infection. The *ΔpldA* mutant showed moderate but not complete recovery in both the antrum and corpus of $C3^{-/-}$ mice, compared to the its performance in WT mice (Fig. 6), but obviously lower than *ΔlctP* mutant in $C3^{-/-}$ mice (Chapter 2, Figure 5). This result showed that *ΔpldA* mutant was not capable of colonizing stomach regions even without the pressure given by complement. The *ΔlctP* mutant was fully recovered in the corpus region of complement deficient mice (Chapter 2, Figure 5), indicating that PldA may play additional roles in *H. pylori* activities not solely as a complement defender.

3.4 Discussion

In this study, we report that L-lactate plays a role as an internal signaling molecule that mediates *H. pylori* complement resistance. By applying a transcriptomics analysis, we discovered that phospholipase A was up-regulated in the presence of L-lactate. We confirmed that PldA was necessary for *H. pylori* complement resistance, acting to destabilize C4b from binding to *H. pylori* surface. If *pldA* is lost, *H. pylori* failed to respond the presence of L-lactate to block C4b deposition. We further found that the loss of *pldA* caused *H. pylori* to have a severe gastric colonization defect, suggesting its importance to *H. pylori* colonization. However, such deficiency was only partially alleviated in complement inactive mice, suggesting that PldA has a role in complement resistance in vivo but may play additional roles in this setting.

Our work suggests that *H. pylori* can use intracellular lactate as a signaling molecule, not only as a carbon source. Compared to other previous studies in other

bacteria, Unique features were found in *H. pylori*. To metabolize L-lactate, *H. pylori* carries two lactate permeases called LctP1 and LctP2 (HP0140 and HP0141), a LutABC-type lactate utilization dehydrogenase called L-LDH or LutABC (HP0137-0139) (Iwatani et al., 2014), which shares the basic principles of the conserved lactate uptake pathway with various bacteria, initially identified in *Bacillus subtilis* and *Shewanella oneidensis* in 2009 (Chai *et al.*, 2009; Pinchuk *et al.*, 2009). Whereases, *H. pylori* carries two nearly identical lactate permease coding genes, which was not observed in other bacteria. This suggests that *H. pylori* may acquire a large amount of lactate during its life cycle, or lactate is an important molecule that *H. pylori* tends to ensure lactate transportation not disrupted. In addition, *lctP* and *L-ldh* of *H. pylori* share different orientation, suggesting that lactate uptake and metabolism may be involved in different regulatory pathways, or have varied function in *H. pylori* activities. Furthermore, no homologs of typical lactate-responsive regulators, GntR, or LysR family, were found in *H. pylori* to regulate lactate metabolism operon. All these evidence supports our finding that lactate plays additional roles in *H. pylori* activities beyond solely as a carbon source. Our RNA-seq data demonstrate L-lactate regulates distinct types of gene expression (Fig. 3A). However, the regulated regulons are small, as only three non-colocalized genes were found upregulated in the presence of lactate. Interestingly, these three genes share no similar sequences in the promoter region, indicating they are regulated by different regulators or under different levels of transcriptional regulation. The future study will focus on exploring how lactate mediates genes expression in *H. pylori*.

The anti-complement mechanism we found in *H. pylori* is unique compared to other resistance mechanisms. *H. pylori* utilizes a strategy that utilizes its own proteins and not those of the host, and blocks C4b deposition, in contrast to other strategies that utilize host regulators to block C4 activation. We initially speculated that *H. pylori* removes the C4b by rapidly shedding the target molecules or employing some type of protease to cleave complement components, like C4. Here, we provide data that suggests *H. pylori* PldA is responsible for C4b removal. PldA is a phospholipase, a type of enzyme best known for cleaving ester bonds formed between alcohol and hydroxyl groups. PldA has not been implicated in complement resistance previously. However, the ester bonds cleaved by phospholipases form the basis for how C4b is covalently bound to the bacterial surface. Our model is thus that PldA cleaves the ester bond formed between C4b and bacterial surface molecules, resulting in lack of stable C4b association.

The ester bond is a key aspect of stable C4b association. This bond is utilized to solidly connect C4b to target cells. One host-based complement inhibitor also cleaves C4b, but does so before it is covalently attached to host cells. This host protein is called Factor I, a serine protease. There are some key differences between phospholipases and typical proteases. First, they function on different substrates. Proteases act on amino bonds, while phospholipases recognize and cleave ester bonds. Bacteria commonly apply proteolytic strategy to directly target and cleave complement component, while the molecules under PldA targeting is still not clear yet. Considering that C4b has a labile internal thioester bond that is exposed during C4 activation process

and reacts broadly with amino and hydroxyl groups (Dodds, 1996), we speculate that PldA either cleaves ester bonds formed between C4b and target molecules of *H. pylori* or directly the ester bonds containing molecules in *H. pylori*, potential targets of C4b. For example, lipooligosaccharide of *Neisseria* was found to be the target of C4b (Ram et al., 2003). The future study will focus on investigating the targets of C4b in *H. pylori* to further elucidate how PldA protect this bacterium from complement mediated cell lysis.

H. pylori ΔpldA mutant was only moderately recovered in $C3^{-/-}$ mice drove us to speculate that PldA may have multiple functions. Indeed, it has been reported that PldA was involved in *H. pylori* pH adaptation (Tannaes, 2001). In that study, PldA was found to be particularly important for survival under acidic conditions. PldA is found in many other bacteria, but the involvement of PldA in pH adaptation has not yet associated with other PldA-containing pathogens. It may be because this questions has not been studied in microbes that live outside the stomach, or because it is a unique property of the *H. pylori* PldA. When searching PldA homologs, we found an interesting variation between PldA proteins of *H. pylori* and enterohepatic bacteria, such as *E. coli* and *Vibrio cholerae*. This difference was that a 33 amino acids peptide was uniquely inserted in *H. pylori* PldA, but not in all examined intestinal bacteria, nor other *Helicobacter* enterohepatic species (data not shown). This observation allowed us to speculate that the inserted peptide may confer additional functions in *H. pylori* PldA, an idea we are actively testing. The involvement of PldA in pH adaption and

complement resistance suggests bacterial pathogens may efficiently adapt to surrounding environment, especially those who has relative small genomes.

3.5 Methods

3.5.1 Bacterial strains and growth condition

H. pylori PMSS1 (Arnold et al., 2011) and isogenic mutant were used for these studies (Table 1). The isogenic *ΔrplU*, *ΔpldA*, and *ΔHPYLPMSS1_0673* mutants were constructed using a PCR-based method as described previously (Chalker et al., 2001). To delete the each of these genes coding genes, the coding regions of all the genes were entirely replaced by a chloramphenicol resistance cassette (*cat*), selected by chloramphenicol (25 μg/ml) and the mutants verified by PCR amplification. For mouse model infections, GFP^+ PMSS1 WT, *ΔlctP*, and *ΔL-ldh* strains were constructed by natural transformation with plasmid pTM115 (Keilberg et al., 2016) to create PMSS1 pTM115 (KO1568) and PMSS1 *ΔlctP* pTM115, and *ΔL-ldh* pTM115. For the transformation, plasmid isolated from *H. pylori* strain SS1 pTM115 was used with selection CHBA plates containing 15 μg/ml kanamycin.

For solid media, *H. pylori* strains were grown on Colombia Horse Blood Agar containing: 5% defibrinated horse blood (Hemostat Laboratories, Dixon, CA), 0.2% β-cyclodextrin, 10 μg/ml vancomycin, 5 μg/ml cefsulodin, 2.5 U/ml polymyxin B, 5 μg/ml trimethoprim, and 8 μg/ml amphotericin B (CHBA) (all chemicals are from Thermo Fisher or Gold Biotech). For antibiotic resistance marker selection, bacterial media was additionally supplemented with 25 μg/ml chloramphenicol or 15μg/ml

kanamycin. For liquid media, *H. pylori* cultures were grown in brucella broth (BB) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (BB10) (Life Technologies). *H. pylori* were grown under microaerobic conditions of 10% CO_2, 5% O_2 and 85% N_2 at 37°C.

3.5.2 Serum resistance assays

H. pylori infection-free normal human serum (NHS, Sigma) was used for *H. pylori* serum sensitivity tests. The NHS was either left untreated (active) or heat-inactivated by heating in a water bath for 60 min at 56 °C. For serum-mediated killing assays, overnight bacterial cultures were diluted to OD_{600} of 0.1 in fresh matched medium (BB with or without lactate), and then NHS (active or inactive) was added to the final volume percentage of 10%. Samples were incubated for 1 hour under microaerobic conditions with shaking. After the desired time, samples were serially diluted and plated on CHBA, and incubated for five days to allow colony formation. Statistical comparisons were made using a two-tail Student's T-test. Differences in *P-value* of < 0.05 were considered significant.

3.5.3 Mouse infection

The University of California, Santa Cruz Institutional Animal Care and Use Committee approved all animal protocols and experiments (Protocol OTTEK1804 and OTTEK2014). Female C57BL/6N mice (Helicobacter-free, Charles River Laboratories), C57BL/6J mice (Helicobacter-free, The Jackson Laboratory) and B6;129S4-C3<tm1Crr>/J mice ($C3^{-/-}$) (Helicobacter-free, The Jackson Laboratory) were housed at the animal facility of the University of California Santa Cruz.

Mice were between 6 to 8 weeks old at the time of *H. pylori* infection. All animals were orally infected via a 20-gauge 1.5-in. feeding needle (Popper) with 100 µl containing ~10^8 GFP^+ *H. pylori* strains. These strains were prepared by liquid growth to late-exponential phase in BB10 medium. After the infection period, the animals were sacrificed via CO_2 narcosis, the stomach dissected, opened along the lesser curvature, and the forestomach region removed and discarded. The remaining tissue was divided into three parts—the corpus, the transition zone, and the antrum, as described previously using difference in tissue coloration as a marker (Rolig et al., 2012). The transition zone was discarded and not included in experimental analysis. Each region was then divided into two pieces with 0.3-0.5 cm^2 of each, one piece for total bacterial number counting and lactate concentration measurement, and the other for gland isolation. For total bacterial number counting, the tissue was weighed, homogenized using the Bullet Blender (Next Advance) with 1.0-mm zirconium silicate beads, diluted and plated to determine the number of CFUs/gram of stomach tissue on CHBA with the addition of 20 μg/ml bacitracin, 10 μg/ml nalidixic acid, and 15 μg/ml kanamycin.

3.5.4 RNA extraction and library construction

WT *H. pylori* was grown 17-18h. A portion of culture was treated by 5 mM L-lactate for 1h before RNA extraction, and the left portion was non-treated. Total RNA of each culture samples were extracted using the Trizol Max Bacterial Enhancement Kit (Ambion, Life Technology, Carlsbad, CA, USA) and purified using RNAeasy Kit (Qiagen) as described by the manufacturer. rRNA was further removed using RiboZero magnetic kit (Illumina). Sequencing libraries were generated using NEBNext

Ultra™ Directional RNA library Prep Kit for Illumina (NEB, USA). Complementary DNA (cDNA) library quality and amount were verified using Agilent Bioanalyzer 2100 system (Agilent technologies, CA, USA) and then sequenced using Illumina NextSeq Mid-Output (UC Davis Genome Center).

3.5.5 Transcriptomics analysis

RNA-seq data was analyzed using CLC Genomics Workbench (Qiagen). With trimming of adapters, forward- and revered-sequenced reads generated for each treatment condition (L-lactate treated or non-treated, with three biological replicates for each condition) were mapped against the PMSS1 reference genome (NCBI-RefSeq: NZ_AP017634). The expression value was measured in reads per kilobase per million mapped reads (RPKM). Genes expression was considered as differentially expressed when the $\log_2$ fold change was above 1 and the *p*-vaule was less than 0.05.

3.5.6 Serum C4 activation assay

Overnight *H. pylori* PMSS1 WT and mutants were suspended into BB media containing ~10^7 bacteria. Active NHS was added to the bacterial culture and allowed to co-incubate for indicated time periods at 37°C. Reactions were stopped by adding 6x sample buffer (0.3M Tris-HCl, pH 6.8, 6 M glycerol, 10% w/v SDS, 9 mM bromophenol blue). Some protein samples were further treated with final 5% β-mercaptoethanol to cleave disulfide bonds. All samples were then incubated at 90°C for 10 min. C4 activation was analyzed by monitoring the formation of cleaved C4 portion, C4b, through western blot.

3.5.7 Western blot

Samples were separated on 5% or 7% (wt/vol) acrylamide SDS/PAGE gels, and then used for immunoblotting. For immunoblotting, gels were soaked in transfer buffer [48 mM Tris-base, 39 mM glycine, 1.3 mM SDS, 20% (vol/vol) methanol] for 10 min and then transferred to an immunoblot polyvinylidene fluoride (PDVF) membrane (Bio-Rad) for 60 min at 25 V through Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad). The membrane was blocked for 1 h with blocking buffer (PBS with 5% milk plus 0.2% Tween-20) at room temperature. Primary antibody was added and incubated for 16-18 h at 4 °C using the 1:1000 dilution for Rabbit anti-human C4 antibody (mAb #60059) (Cell Signaling). After incubation, the membranes were washed and HRP-conjugated Goat anti-rabbit (Santa Cruz Biotechnology) added at 1:10,000. After incubation, the membranes were washed and treated with HRP substrates (SuperSignal West, Pico and hydrogen peroxide mixed at 1:1 ratio; Thermo Scientific). Blots were then visualized using a Chemidoc imaging system (Bio-Rad).

Figures

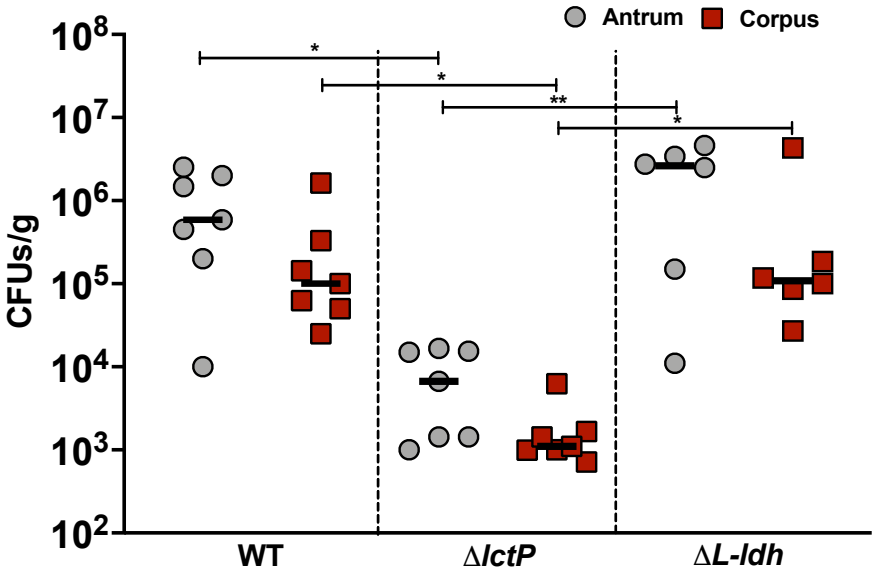


Figure 3.1 *L-ldh* mutant colonizes mouse stomach to normal levels during early infection

C57Bl6/N mice were infected by oral gavage with GFP$^+$ *H. pylori* PMSS1 WT, *ΔlctP* or *ΔL-ldh* mutant for two weeks. Stomachs were isolated from infected mice and separated into corpus and antrum pieces. A tissue piece from each region was homogenized and plated to obtain the total colony forming units (CFUs) from antrum (gray) or corpus (red) normalized to tissue weight (CFUs/g). CFU data of WT and *ΔlctP* mutant is identical to it of Chapter 2, Supplemental Figure 5. Statistical analyses were performed using one-way ANOVA with Tukey post hoc test, indicated as * ($P < 0.05$), ** ($P < 0.01$).

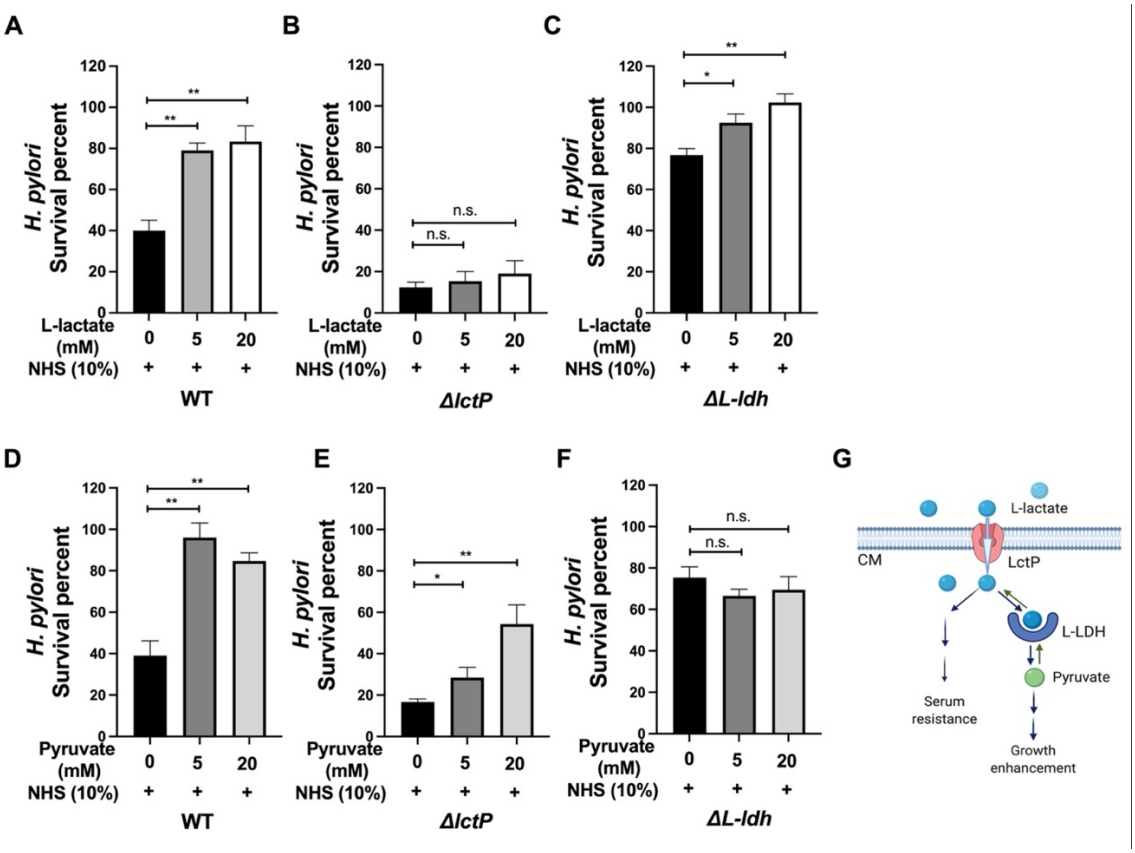
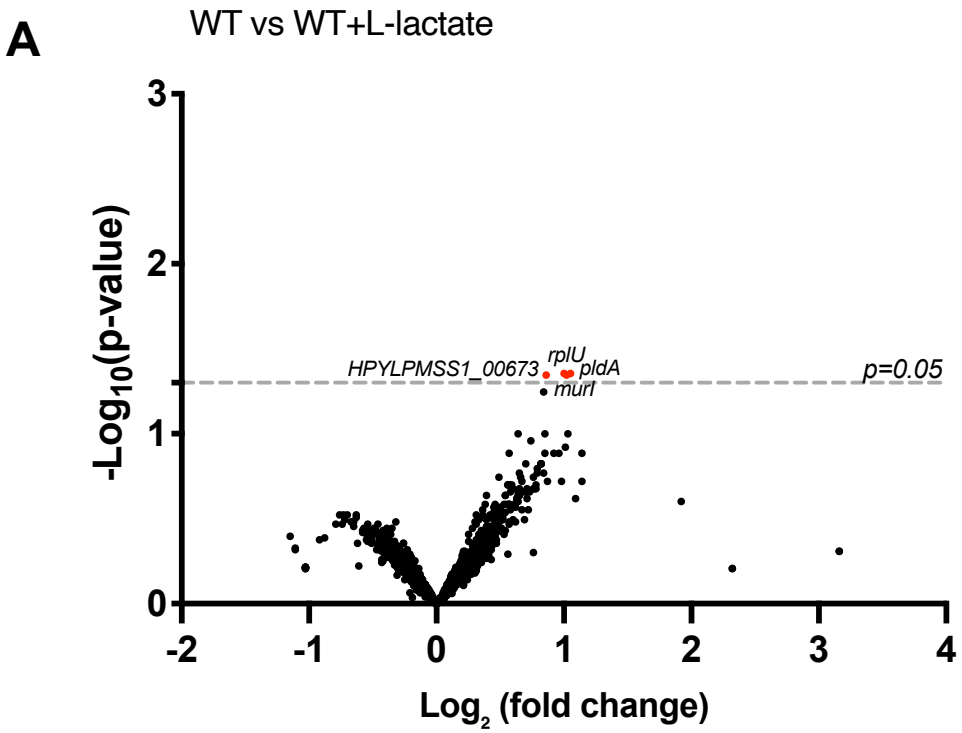


Figure 3.2 Lactate mediated complement resistance does not depend on lactate metabolism

H. pylori PMSS1 WT, or isogenic *ΔlctP*, and *ΔL-ldh* mutants were grown overnight with the supplementations of indicated molecules, and then treated by active or heated inactive NHS. The survival percent was determined by dividing the CFUs of active NHS treatment by total CFUs obtained from inactive NHS treatment. (A-C) PMSS1 WT, *ΔlctP* and *ΔL-ldh* cultures, supplemented with indicated concentration of L-lactate, were exposed in 10% NHS for 1h. (D-F) PMSS1 WT, *ΔlctP* and *ΔL-ldh* cultures, supplemented with indicated concentration of pyruvate, were exposed in 10% NHS for

1h. (G) Proposed model for L-lactate mediated complement resistance. All results were presented as survival percent ± standard deviation (SD), and were derived from triplicate biological samples with a triplicate of each given sample. The p-values were obtained by one-way ANOVA with Tukey post hoc test. The significance was indicated as * (P<0.05) and ** (P<0.01). The n.s. indicates no statistical significance compared to no treatment control.



B

Locus	Description	Fold change
pldA (HP0499)	Phospholipase A	2.1
rplU (HP0296)	50S ribosomal protein	2.03
murI (HP0549)	Glutamate racemase	2

Figure 3.3 Lactate regulates transcriptional expression of several genes

Overnight culture of *H. pylori* PMSS1 WT were either treated with 5mM L-lactate for 1h or left as untreated before total RNA extraction. rRNA was depleted and mRNA was analyzed by RNA-seq. (A) The volcano plot represents gene expression. The y axis is the negative $\log_{10}$ of *p*-values, and the x axis is the log2 fold change gene abundance between two population (positive values represent the upregulated genes in the presence of lactate, and negative values represent downregulated genes). The dashed line indicated the position that $p = 0.05$. The dots above this line were considered as significantly differentially expressed genes. (B) The upregulated genes when *H. pylori* responds the presence of L-lactate. Upregulation was determined as a cutoff ratio of ≥1 log2 fold (2-fold) change and *p*-value of <0.05.

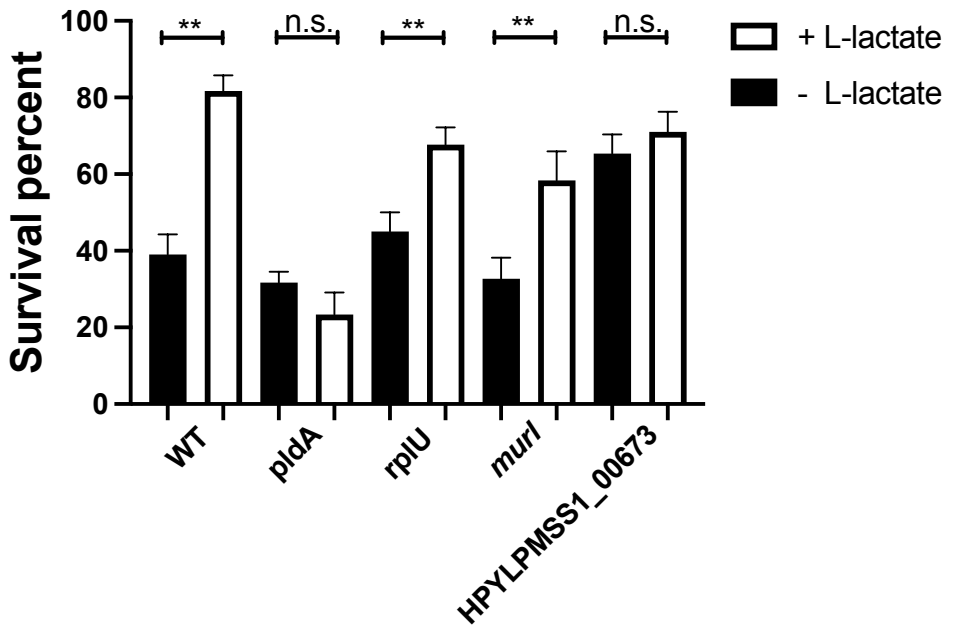


Figure 3.4 Complement resistance of Phospholipase A was not promoted by the presence of lactate

Complement sensitivity was tested for *H. pylori* isogenic mutants that are significantly upregulated in the presence of lactate from RNA-seq data. Overnight cultures of *H. pylori* PMSS1 mutants were treated by inactive and active NHS with final percentages of 10% for one hour, and then viable bacterial CFUs were determined by plating. The survival percent was determined by comparing the number of CFUs from active serum divided by the CFUs from heat-inactivated serum treatment. For the survival ratio of each mutant grown with or without L-lactate, results were presented as survival percent ± standard deviation (SD), and were derived from triplicate biological samples with a

triplicate of each given sample. The p-values were obtained with a two-tail student *t*-test. The significance was indicated as ** ($P<0.01$), or n.s. (not significant).

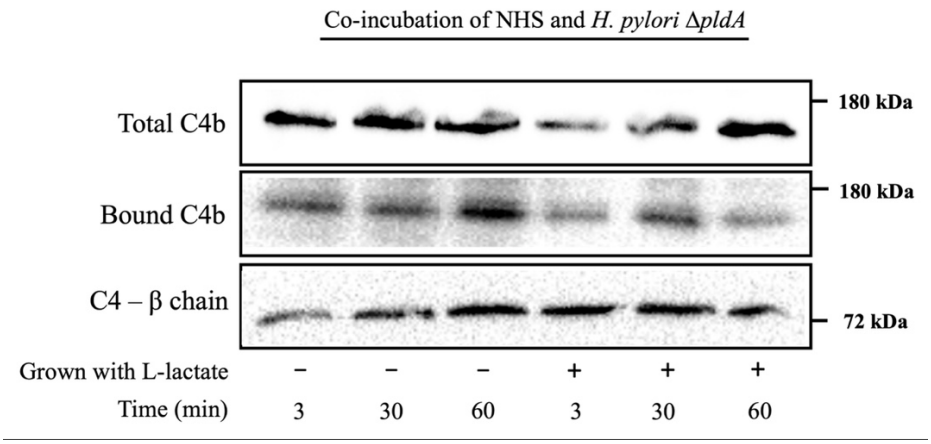


Figure 3.5 Phospholipase A is indued by L-lactate to destabilizing C4b from H. pylori surface

H. pylori PMSS1 *ΔpldA* mutant was grown ± L-lactate and then treated by 10% NHS for the indicated time periods. Protein samples were prepared and blotted as for WT *H. pylori* showed in Chapter 2 Figure 4D.

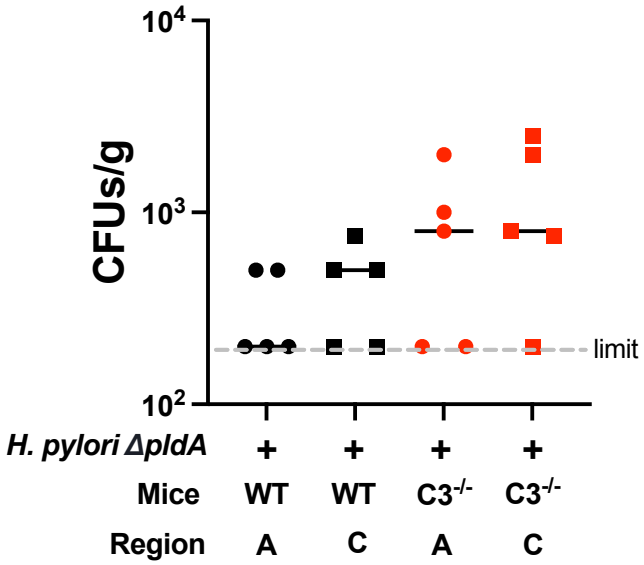


Figure 3.6 pldA mutants have a strong mouse colonization defect that is moderately recovered in complement deficient mice.

C57Bl6/J WT or $C3^{-/-}$ mice were infected by oral gavage with GFP^+ *H. pylori* PMSS1 for two weeks. Stomachs were isolated from infected mice and separated into corpus and antrum pieces and processed to obtain the total bacterial numbers (colony forming units, CFUs) by plating. Total bacterial number from gastric antrum (dots) and corpus (square) of infected WT or $C3^{-/-}$ mice normalized to tissue weight (CFUs/g). Gray dotted line, limit of detection. The *p*-values for multi sample comparison were obtained by one-way ANOVA (Tukey's test), and no significant differences were found.

Strain	description	Source(s)
PMSS1	WT strain	Arnold et al., 2011
PMSS1 *ΔlctP*	*Δhp0140-0141::cat*	This study
PMSS1 *ΔL-ldh*	*Δhp0137-0139::cat*	This study
PMSS1 pTM115	GFP^+ PMSS1 WT	This study
PMSS1 *ΔlctP* pTM115	GFP^+ PMSS1 *ΔlctP*	This study
PMSS1 *ΔL-ldh* pTM115	GFP^+ PMSS1 *ΔL-ldh*	This study
PMSS1 *ΔpldA*	*Δhp0499::cat*	This study
PMSS1 *ΔrplU*	*Δhp0296::cat*	This study
PMSS1 *ΔmurI*	*Δhp0549::cat*	This study
PMSS1 *ΔHPYLPMSS1_00673*	*ΔHPYLPMSS1_00673::cat*	This study

Supplemental Table 3.1 Strains used for this study

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Chapter 4

Evasion of the restriction-modification system significantly elevate bacterial genetic manipulation efficiency

4.1 Abstract

Genetic manipulation is a frequently applied approach to study numerous bacterial processes. However, many bacteria are difficult to manipulate, at least in part due to robust Restriction-Modification (RM) systems that destroy exogenous incoming DNA. Here, we report an easily applicable approach to overcome this barrier and improve bacterial transformation efficiency. For this approach, we computationally predicted under-represented short Kmer sequences in the *H. pylori* genome, with the idea that these sequences reflect restriction enzyme targets. We then used this information to modify an antibiotic resistance cassette by generating synonymous mutations at the predicted restriction sites, and used this modified cassette for transformation. Indeed, antibiotic cassettes with modified under-represented Kmer sites resulted in up to 10^5-fold higher transformation efficiency compared to a non-modified cassettes. We also show that use of conditions under which RM systems are expressed to low levels further enhances transformation efficiency by ~ 5-fold. Because a great enhancement can be obtained by Kmer site prediction, this approach can be readily applied to any microbe with a sequenced genome. We thus expect this approach will enable genetic manipulation to be more achievable in a wide range of bacterial species.

4.2 Introduction

Horizontal gene transfer (HGT) is a key driver of bacterial evolution. During HGT, new DNA is taken up and integrated into a recipient bacterium's genome. Bacteria utilize HGT for the adaptation to new environments, antibacterial resistance and the spread of virulence factors. Furthermore, identifying horizontally acquired genes opens a window to explore fundamental aspects of bacterial physiology, metabolism, and pathogenesis. In addition, HGT-inspired approaches are used by scientists to carry out bacterial genetic manipulation. A common approach is to use natural transformation, in which free DNA is taken up and maintained in the recipient cell. However, the feasibility of natural transformation depends on bacterial competence levels, including the ability to protect the incoming DNA from host defense mechanisms (Rocha & Bikard, 2022; Ando et al., 2000). As scientists strive to genetically manipulate more and more bacterial species, efficient tools are highly needed.

After DNA uptake, DNA integrity is affected by the bacterial restriction-modification (R-M) systems (REF). R-M systems are one of the most widespread bacterial systems that enable bacteria to distinguish self- from non-self DNA. These systems consist of two enzymes: a restriction endonuclease (REase) and a methyltransferase (MTase) (Oliveira, 2014). The REase functions to patrol and degrade unmethylated or non-host methylated DNA at highly specific and conserved sequences, while the MTase functions to methylate DNA sequences at particular sites to evade REase recognition and digestion (Mruk, 2014). There are numerous R-M systems,

recognizing over 450 motifs that have different patterns. The REases themselves are divided into four types (type I, II, III, and IV) (Donahue & Peek, 2001; Loenen et al., 2014). In some cases the REase site can be identified. Type II and III REases cleave DNA at a defined position close to or within their recognition sequences. Type I REases, however, cleave DNA at random distance from their recognition sequences, making their sites challenging to predict (Loenen et al., 2014). Type IV, an atypical type of R-M systems, exclusively cleave modified but not naked DNA (Loenen & Raleigh, 2014). REase digestion limits what sequences can be horizontally acquired, and so it would be beneficial to develop a broadly applicable strategy that can overcome RM barriers of given bacteria.

The RM system likely initially evolved to protect against invading external DNA, which is primarily from phages. Phages have been observed to have sequences that avoid restriction sites (Zabeau, 1980; Tock, 2005). Consequently, various approaches have been developed to overcome the restriction by the RM systems that are inspired by phage anti-restriction mechanisms. One strategy is to methylate the exogenous DNA to match the host's, via in vitro methylation using purified MTases or a crude extract of the target bacterium (Donahue et al., 2000; Zhang et al., 2012). This method has been used in some bacterial transformation, such as *Bacillus weihenstephanensis* and *Helicobacter pylori* (Donahue et al., 2000; Zhang et al., 2012). It is low-cost and versatile for multipurpose genetic modification, but has challenges since not all enzymes are readily available in pure forms, the enzymatic activity of methyltransferase in crude extract is not consistent, and there are competing DNAses

that lower the overall DNA amount (Bergsmedh et al., 2006). Another strategy is to subvert the restriction-modification activities by either stimulating host MTase or eliminating REase via genetic engineering (Zhang et al., 2012). Deletion of REases coding genes is one common strategy to alleviate the restriction of external DNA. This method can increase horizontal gene transfer (HGT) efficiency but it may be difficult to identify and delete all REases.

REases bind specific DNA sequences to target them for cutting. These sites are typically 4-6 basepairs in length. Having these sites present on the genome is dangerous, because they could be cut and cause DNA damage when unmethylated, e.g. after replication. Thus, it would evolutionarily advantageous to avoid these sites. Indeed, Rocha *et al*. reported that palindromes with half sites of size 4 to 6 bases are under-represented in some of the prokaryotic genomes, (Rocha et al, 2001). Those palindromic sequences could be targets of type II restriction enzymes. This concept can be expanded to all under-represented short DNA sequences, not only palindromes, because Type III and IV RE are able to recognize non-palindromic DNA sequences (Meisel et al., 1992; Loenen & Raleigh, 2014). We, therefore, were encouraged by an easily testable hypothesis: if a given DNA lacks the short DNA sequences that are under-represented in the target bacterial genome, it will be immune to REases recognition and destruction. This avoidance, in turn, would be predicted to increase transformation efficiency. These under-represented motifs can be systematically predicted from the genome sequences, and then modified on a given DNA sequence by synonymous nucleotide substitution.

We report here that the efficiency of the transformation was remarkably increased by applying the above approach in the bacterial pathogen *Helicobacter pylori*, as a demonstration of the proof-of-principle. We furthermore show that under conditions that REases are less expressed transformation efficiency was further increased. *Helicobacter pylori* is gram-negative bacterium that can persistently colonize the human stomach, dominant among gastric microbial populations, and is the key factor for development of gastric ulcers and cancers. There is great interest in understanding the mechanisms of *H. pylori* induced diseases. *H. pylori* is naturally competent but often has low transformation efficiency with exogenous DNA. Thus what we learn with *H. pylori* will benefit important work with this microbe and also pave the way for applying our principles for efficient genetic manipulation strategies.

4.3 Results

4.3.1 Under-represented short DNA sequences can be identified in the *H. pylori* genome

To predict potential REs recognition DNA motifs, we screened for short DNA sequences that occur significantly less frequently than would be expected. We set up a null model as any nucleotide at position k of a genome would have same probability presented and applied simple Markov model to screen for significant under-represented motifs with E-value < 0.01 (false positive with less than 0.01 probability). We designed our model that does not restrict any length of short sequences to allowed us to search any under-represented short sequences. Based on the REs recognition pattern about short DNA length, we set up 4-6 nucleotides at position k as a subpopulation each time

to calculate their present probability. We applied this analysis to the *H. pylor*i SS1 genome and found that there are 42 under-represented Kmer sequences (Table 1). We further found that the direction of sequences extending from a position k does not impact the final statistical results, since either asymmetry of extending in both directions from the central or one direction from a start position gained us capacity to identify the same short sequences list (Table 1). We also applied this Markov model screen to a wide set of different *H. pylori* strains. These strains all shared the same under-represented short sequences (Table 1), supporting the idea that these under-represented sequences are consistent across a species, likely the result of a long evolutionary period and not due to recent strain-specific activity.

4.3.2 Modification on antibiotic resistant cassette

We next created a version of a standard antibiotic resistance gene cassette that lacked the 42 underrepresented sites. We focused only on the coding region of the antibiotic resistant gene, to avoid any changes to transcriptional control. We chose the commonly utilized chloramphenicol resistant gene (*cat*), from *Campylobacter coli* (Wang et al., 1990), for modification by changing predicted underrepresented Kmer sites with synonymous mutations to alter these sites without altering the protein amino acid sequence (Fig. 1). After designing these modified cat genes, they were ordered as synthetic genes. We focused only on the *cat* cassette since *H. pylori* performs double crossover recombination and thus the plasmid backbone does not participate.

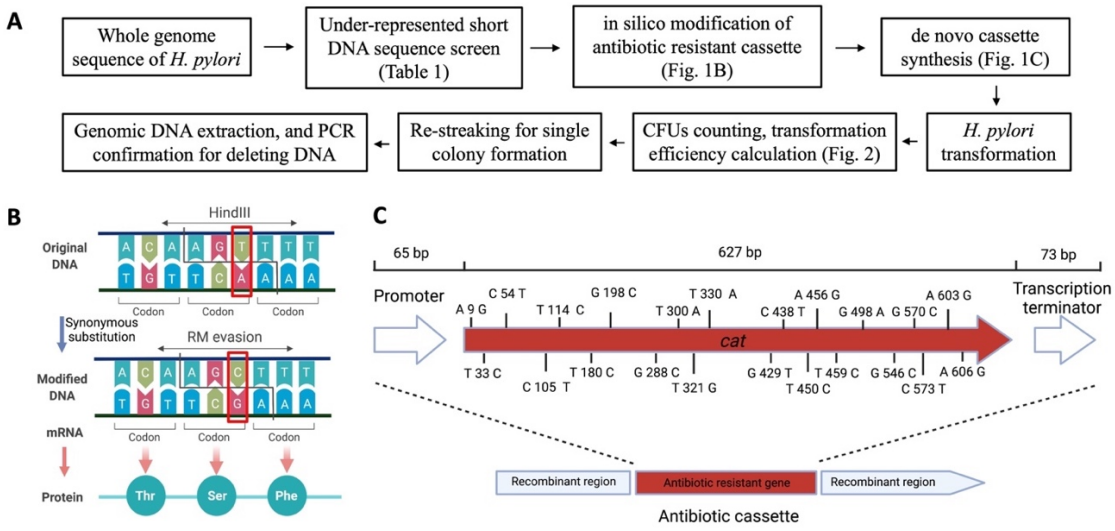


Figure 4.1 Schematic representation of under-represented sequence motif based DNA modification and bacterial transformation.

Strategy for potential restriction sites removal based gene modification and transformation. (A) Workflow for under-represented Kmer identification, antibiotic gene modification and target gene deletion. (B) Example for identification of subpopulations harboring restriction recognition-motifs and strategy of gene modification. Antibiotic resistance gene (*cat*) sequence was compared against predicted under-represented motifs list (Table 1). The relative coded amino acid sequence was also checked. The illustration shows the main modification strategy as synonymous substitution of the third nucleotide of a codon in the RE recognition-motif. (C) Schematic of the modified cat gene (cat_{Kmer}), with 22 nucleotides synonymously substituted based on Kmer prediction. The recombinant region could be any region of the target gene based on experimental purpose.

4.3.3 Transformation efficiency is significantly elevated with engineered antibiotic cassette

Next, we wondered whether our modified *cat* cassette gains a significant improved transformation efficiency. We aimed to delete a *H. pylori* gene to test our hypothesis. Lactate permease coding genes (*lctP 1-2*, *hp0140-0141*) (Iwatani et al., 2014) were chosen since lactate utilization was recently proposed to be important for this bacterial gastric infection (Keilberg et al., 2021). We thus created a construct that flanked a WT *cat* gene with 500 basepairs upstream of *lctP1* and 500 basepairs downstream of *lctP*2 (Supplemental Fig. 1). This construct, however, transformed poorly (Fig. 2A). The transformation efficiency was estimated to be lower than 10^{11}cells/µg DNA. We then created a second construct, which replaced the WT *cat* allele with cat_{Kmer} (Fig. 1C) and retained the same *lctP1* and *lctP2* flanking sequences (Supplemental Fig. 1). This construct resulted in significantly improved transformation efficiency by more than 1.9 x 10^5-fold in comparison to non-modified cassette (Fig. 2A). This result suggests that removal of possible restriction sites can have a dramatic effect on transformation efficiency.

4.3.4 Surplus of RE-sites removement did not gain an advanced transformation efficiency

Next, we questioned whether targeted removal of predicted RE sites would further increase the transformation efficiency of the *cat* cassette. We again examined the *cat* gene, and identified known *H. pylori* RE sites using the NEB Rebase database, regardless of whether sites were identified as under-represented or not. 19 target

sequences were predicted as the potential targets of REs (Table 2). We then further modified the *cat*$_{\text{Kmer}}$ by editing sequences corresponding to these RE, making synonymous substitutions. We tested this cassette, called *cat*$_{\text{RE+Kmer}}$ with the same *lctP1-2* flanking sequences as before (Supplemental Fig. 1). Unexpectedly, *cat*$_{\text{RE+Kmer}}$ did not exhibit improved transformation efficiency (Fig. 2A). It was actually relatively less efficient than Kmer only modified cassette, about 4,600-fold improved from non-modified cassette (Fig. 2A). These outcomes suggest that modification of Kmer sequences only is most effective at improving external DNA transformation efficiency.

4.3.5 Kmer based antibiotic cassette modification exhibits consistent recombinant substitution capacity in different *H. pylori* genomic loci

We next examined whether the Kmer modified cassette would similarly enhance transformation of other genes. We chose the L-lactate dehydrogenase coding genes (*L-ldh*, *hp0137-0139*), another essential enzyme for lactate utilization (Iwatani et al., 2014). In this process, *H. pylori* transformed with the non-modified *cat* cassette showed efficiency at 10^{-9} CFUs/µg DNA level (Fig. 2B), about 100-fold higher than it was for *lctP1-2* deletion (Fig. 2A). This result suggests that local genomic or genetic variations can cause varied outcomes of external DNA transformation. Transformation with the *cat*$_{\text{Kmer}}$ modified antibiotic cassette again resulted in greatly enhanced transformation compared to unmodified WT *cat*, yielding 10^{-5} CFUs/µg, a 1600-fold increase (Fig. 2A, 2B). These outcomes suggested that the Kmer only based transformation is broadly efficient.

4.3.6 Revised transformation procedure improved transformation for non-modified cassette but not for Kmer modified cassette

From our previous research, we noticed that some of the restriction enzyme coding genes in *H. pylori* were up-regulated during biofilm growth style versus the planktonic form (Hathroubi et al., 2018, 2020). This observation was intriguing, because *H. pylori* transformations are normally done on plate-grown cells, which could mimic a biofilm state. We therefore wondered whether alternative culture methods, e.g. liquid culture, would elevate *H. pylori* natural transformation. To test this idea, we carried out transformations with liquid, planktonic bacteria (referred to as liquid-based method) instead of using plate-growing bacteria. The efficiency of transformation with non-modified WT *cat* cassette for the *lctP1-2* allele jumped from non-detectable (less than 10^{-11} CFUs/µg DNA) (Fig. 2A) to above 10^{-9} CFUs/µg DNA (Fig. 2C) by applying this derived liquid-based method. A similar trend was observed as well in the *L-ldh* targeted transformation, 23-fold elevated (Fig. 2B). These results suggest that this derived approach promotes *H. pylori* transformations. We next evaluated transformation efficiency of the Kmer modified *cat* cassette in this liquid-based method. Again, we saw a strong improvement of about 630 to 2800-fold when using the Kmer modified cat (Fig. 2B, 2C). Such consistency suggests that our Kmer only modified cassette based transformation may be close to the optimal transformation levels.

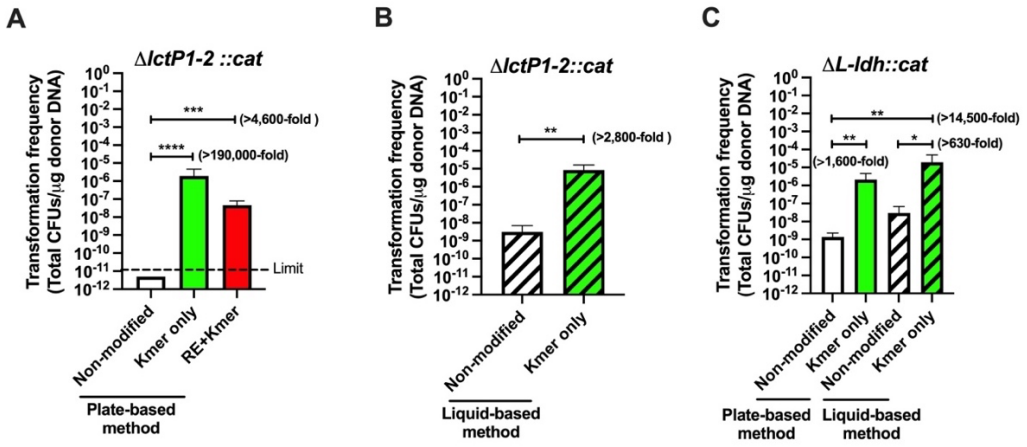


Figure 4.2 Engineered antibiotic cassettes significantly improve *H. pylori* transformation efficiency.

Transformation efficiency was measured by determining the number of chloramphenicol-resistant colony forming units per μg of DNA. (A) Target gene transformation efficiencies were compared between Δ*lctP1-2*::*cat* cassettes that were non-modified (WT), Kmer only, or RE+Kmer modified. The plate-based transformation procedure was performed in this experiment. (B) Transformation efficiency with Δ*L-ldh::cat* comparing the WT cat and Kmer only, using either plate-based or liquid-based method. (C) Same as panel A, but using the liquid-based transformation method. All the results are presented as transformation efficiency ± standard deviation (SD), and were derived from triplicate biological samples with technical triplicates of each given sample. The *p*-values were obtained with a two-tail student *t*-test. The significance is indicated as * (P<0.05), ** (P<0.01), *** (P<0.001), and **** (P<0.0001).

4.4 Discussion

In this study, we report two approaches for improved microbial genetic engineering by facilitating evasion of RM systems. The first approach was based on a theory that genomes evolve to eliminate restriction sites, resulting in under-representation of these sequences. We used this significant feature to predict potential RE sites by simply determining those under-represented sequence motifs on target bacterial genome. This approach is relatively simple and not labor intensive. This under-represented Kmers based DNA modification approach used in this study can target any gene of *H. pylori*, and simply requires identifying the kmer's in the foreign DNA of interest, in this case *cat*, and creating synonymous mutations to alter the Kmer sites.

A similar strategy to evade external DNA from recognition of RM system was utilized in *Staphylococcus aureus* (Johnston et al., 2019). Their approach differs in how they identified possible REase sites. These scientists determined the sequence motifs protected by the MTases via applying a single-molecule real-time genome and methylome sequencing, and further inferred the methylation protected motifs would be recognized and degraded by their cognate REases. In comparison, we identified potential REases recognition motifs directly by screening under-represented short DNA sequences. That approach requires different types of sequencing, while our approach needs only the information of whole genome sequence of the target bacterium. With the development of genomic sequencing and accumulated knowledge in those few bacterial model systems, reverse-genetic approach become more and more crucial to expand knowledge to largely unknown microbial community. However, it is

commonly impaired by robust bacterial defensive barrier, RM systems. Our approach provides an easily and efficient route to overcome the restriction of RM systems that facilitate such transitions. demonstrated our approach is appliable to a broader range of microbial community.

Beside mutating external DNA to overcome RM barrier recognition, we optimized our classical transformation procedure as well to promote bacterial transformation by positioning *H. pylori* into the environment that naturally suppresses REases expression. This liquid-based transformation idea was inspired by the observation that several REases, such as HypAV, HpyAIV, and R.Pab1 are significantly up-regulated in biofilm growth condition (Hathroubi et al., 2018, 2021). Several other REases coding genes showed an upregulation trends as well. By confirming the proficiency of this optimization, we conclude that this procedure is suitable as the supplementation of REs sites modification approach, individually or synergistically overcome *H. pylori* R-M barriers. We envision that positioning bacteria in their REases down-regulated environment would also be likely to elevate their natural transformation competence.

Our two approaches are complementary. The Kmer approach requires modified genes, but results in very high transformation efficiency. The modified liquid method can be used with any existing genetic construct. Together, they will greatly benefit *H. pylori* genetic engineering and may also apply to other bacteria with robust RM systems.

4.5 Materials and Methods

4.5.1 Strains and growth conditions

H. pylori wild-type strain PMSS1 (Arnold et al., 2012) and *E. coli* DH10B (NEB) were used in this study. *H. pylori* strains were grown on Colombia Horse Blood Agar containing: 5% defibrinated horse blood (Hemostat Laboratories, Dixon, CA), 0.2% β-cyclodextrin, 10 μg/ml vancomycin, 5 μg/ml cefsulodin, 2.5 U/ml polymyxin B, 5 μg/ml trimethoprim, and 8 μg/ml amphotericin B (CHBA) (all chemicals are from Thermo Fisher or Gold Biotech). For liquid media, *H. pylori* cultures were grown in brucella broth (BB) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (BB10) (Life Technologies). *H. pylori* were grown under microaerobic conditions of 10% CO_2, 5% O_2 and 85% N_2 at 37°C. *E. coli* DH10B strain was used for maintain de novo synthesized non-modified (WT), Kmer only modified (cat_{Kmer}), and RE+Kmer modified ($cat_{RE+Kmer}$) cassettes. The stock *E. coli* strains were maintained in Luria-Bertani (LB) broth plus 30% final concentration of glycerol (Fisher) in -80 °C. For liquid culture, each *E.coli* stain was inoculated in LB broth and grown in aerobic condition at 37°C.

4.5.2 Under-represented Kmer analysis

To compute the probability that an arbitrary n-mer is the word (W) that we are interested in, we will use 3 components: the probability that the core (n-2) bases match, and the probabilities of the first and last bases given that the core matches.

$$P(W) = P(W_1 \mid W_2...W_{n-1}) * P(W_n \mid W_2...W_{n-1}) * P(W_2...W_{n-1}) .$$

If we check for the palindrome N times (roughly the length of the genome), we would expect to see N*P(W) occurrences of W. We can use counts and maximum-likelihood estimates of the probabilities in our null model to get an estimation formula:

$E(C(W)) = C(W_1...W_{n-1}X) * C(XW_2...W_n) / C(XW_2...W_{n-1}X)$,

where E(C(W)) is the expected value for the count of the number of times W occurs in the genome, and $C(W_i...W_j)$ is the actual count of the number of times the word $W_i...W_j$ occurs. The 'X' character on the subwords is intended to represent "any character". We are counting the number of words of length n that agree on the first n-1, the second n-1, or the middle n-2 positions. In practice, since our genome sequences are very long, we can ignore edge effects and just use

$E(C(W)) = C(W_1...W_{n-1}) * C(W_2...W_n) / C(W_2...W_{n-1})$,

If n=2, we are not counting empty strings on the bottom, but the number of 2-letter windows in our training data. (Actually, I counted the number of letters, which is very slightly larger.)

Because our model for the count is the sum of N almost independent observations, each with probability P(W), it can be well modeled as a binomial distribution, with variance

$Var(C(W)) = N* P(W) * (1-P(W)) = E(C(W)) * (1 - E(C(W))/N)$, and the standard deviation is

sigma(W) = sqrt(E(C(W)) * (1 - E(C(W))/N)) . Note: because E(C(W)) is much smaller than N, the variance is approximately the same as the mean.

We can then compute a Z-score for each of our words:

Z(W) = (C(W) – E(C(W))) / sigma(W) . Using a Z-score is quite reasonable here, because we are expecting a distribution very close to a normal distribution. (Also, a grad student did an implementation with exact binomial distributions and got almost the same results as with the normal approximation.)

4.5.3 Construction of modified antibiotic resistant gene

We generated two sets of sequences to remove from the target genes. First, the 41 most significantly underrepresented Kmers were collected (Table 1). Second, additional possible restriction sites were identified using genomic predictions. The *H. pylori* SS1 genome (GenBank accession number CP009259) annotations were queried for the terms “restriction” or “methylase”. Each encoded protein sequence was BLASTed at the NEB Rebase (http://rebase.neb.com/rebase/rebase.html) to determine the closest matches and predicted restriction sites. This effort resulted in 19 predicted restriction enzymes, of which 11 had good quality recognition site predictions (Table 2). To facilitate finding these motif sequences in the target genes, we matched each sequence to a restriction enzyme, and then used common restriction enzyme finders in Geneious (Biomatters) to find those motif sequences in the target gene. The genes used were Campylobacter coli cat (REF). After finding the sequences, we then manually edited to alter the third base of a codon to eliminate the restriction site without altering

the amino acid produced, except in rare cases where the amino acids were changed to conserved residues.

4.5.4 *In silico* and *de novo* assembly of antibiotic resistant cassettes

To in silico assemble antibiotic resistant cassette for *lctP1-2* targeted transformation, 500 basepairs upstream of *lctP1* (*hp0140*) and 500 basepairs downstream of *lctP*2 (*hp0141*) were flanked with isogenic *cat* genes, WT, cat_{Kmer}, and $cat_{\text{RE+Kmer}}$ respectively (Supplemental figure 1). The cassettes were then commercially synthesized (Biobasic), and maintained in pUC59 plasmid vectors, called pUC59-cat_{wt}, pUC59-cat_{Kmer}, and pUC59- $cat_{\text{RE+Kmer}}$. For long term conservation and amplification, antibiotic cassettes contained plasmids were transformed (NEB provided protocol) into *E. coli* DH10B strain and conserved in -80 °C. For *L-ldh* targeted transformation, 500 basepairs upstream of *hp0137* and 500 basepairs downstream of *hp0139* were PCR amplified and flanked with isogenic *cat* genes, WT, cat_{Kmer}, and $cat_{\text{RE+Kmer}}$ respectively (Table 4) (Supplemental figure 1). The same concentration of PCR products for *L-ldh* and the plasmids for *lctP* (equal antibiotic cassette weight) were transformed into *H. pylori* WT strain.

4.5.5 Transformation assay

For classical plate-based method, a large loop of 1 day old *H. pylori* from maintained CHBA plate was inoculated onto a new CHBA plate for 5 hours. Then 5 µg of DNA of antibiotic cassette was added to the top of the inoculated bacteria. The mixture was continuously incubated for 18-24h in microaerobic conditions.

For the derived liquid-based method, *H. pylori* was grown on a CHBA plate for 24 hours. Then a large loop of bacteria from CHBA plate was resuspended in BB10 and incubated for 0.5-1h microaerobic conditions. 5 µg of DNA of antibiotic cassette was then added to resuspended *H. pylori* culture. The tubes were incubated for 5 minutes at room temperature, then transferred to a CHBA plate for additional 18-24 hours incubation in microaerobic condition. To the end of incubation period, the transformants containing bacteria with either classical or derived method were collected from the plate and resuspended in BB10. Serial dilutions were applied for plating on antibiotic (Cm) selective plate or CHBA plate. Plates were incubated in microaerobic condition for 4-6 days to allow bacterial colonies formation. Transformation efficiency for each experiment was calculated as (CFU x dilution factor on selective plate)/(CFU x dilution factor on CHBA plate)/5µg DNA.

Tables

Table 4.1 Under-represented Kmer sequences in *H. pylori* strain SS1 and G27

Kmer	observed	expected	E_value	Enzyme that cuts this site
ACGT	210	3081.7626	0.00E+00	HpyF13III
CGCG	2601	6035.9191	0.00E+00	HpyF52II
GCGC	6141	10073.1548	0.00E+00	HhaI
TCGA	311	2452.5424	0.00E+00	TaqI
AGCT	6662	10331.0002	6.40E-284	AluI

GGCC	1568	3783.5414	1.57E-281	HaeIII
GAXTC	2735	5233.6002	5.29E-259	HinFI
ACXGT	561	2051.8156	1.26E-234	HpyF121
CTXAG	3004	5254.2974	4.80E-209	HpyF11I
GTAC	131	1168.0321	3.73E-199	RsaI
GCXGC	2474	3682.8067	3.00E-85	Fsp4HI
GGXCC	861	1614.8887	2.28E-75	Sau96I
GATATC	30	399.6331	4.03E-73	EcoRV
GAATTC	178	619.3197	3.69E-67	EcoRI
ATAT	5781	7201.7192	5.93E-60	None
CTAG	4176	5400.2864	2.73E-59	Rma485I
TCXGA	1304	2024.525	1.48E-54	Hpy188I
GCGCGC	276	664.2976	4.42E-48	Uba69I
AATT	14338	16221.4519	9.77E-47	TseCI
CGXCG	1291	1940.4668	5.03E-46	Hpy991, cuts CGWCG
GATC	5243	6186.6023	4.75E-30	BstXI

GAGCTC	94	286.2624	1.07E-26	SacI
GTTAAC	29	179.385	4.99E-26	HpaI
GCTAGC	266	505.546	2.79E-23	BmtI
TCTAGA	158	350.1145	1.66E-21	XbaI
CGCGCG	114	281.361	3.21E-20	None
TCGCGA	11	111.2265	3.44E-18	SpoI
CAXTG	4543	5214.8755	1.99E-17	None
TTAA	18340	19616.9465	7.81E-17	MseI
CCTAGG	71	184.0625	1.32E-13	BlnI
GGCGCC	32	114.7566	1.89E-11	KasI
GTGCAC	42	129.6326	2.36E-11	DaqI
CCXGG	1423	1734.2702	1.28E-10	HpyNI
TAATTA	343	511.6844	1.49E-10	PacI (TTAATTAA)
GTATAC	15	80.9183	3.96E-10	SnaI
AGXCT	5955	6542.2644	5.86E-10	None
CCATGG	224	361.8336	7.24E-10	NcoI

AAXTT	15862	16767.2496	3.57E-09	None
GGATCC	107	205.3012	1.16E-08	BamHI
GTXAC	1705	2012.0395	1.26E-08	MaeIII
TCATGA	281	418.7449	2.83E-08	BspHI

Kmer sequences that are underrepresented in H. pylori strain SS1 and G27. Observed is the number of times this sequence appeared in the genome; expected is the number of times this sequence was predicted. E_value is the significance; enzymes column indicates an enzyme that cuts this site that was used to locate the sequences in the target genes.

Table 4.2 Predicted restriction enzymes in the *H. pylori* SS1 genome

Gene/Name	Type	Predicted Site	Geneious
HPYLSS1_00089/MboR1	II	GATC	Yes (BxtXII)*
HPYLSS1_00343 (TauI type)	II	GNSGC	Yes (TauI)
HPYLSS1_00356	IV	Un	No
HPYLSS1_00429	I	Un	No
HPYLSS1_00447	II	AATAAT	Yes (VspI)
HPYLSS1_00450 (Hp2012 type)	II	ACNGT	Yes (HpyF12I)
HPYLSS1_00487	II	GGCC	Yes (HaeIII)
HPYLSS1_00494	I	Un	No
HPYLSS1_02960	I	Un	No
HPYLSS1_00656	II	GTNNAC	Yes (MjaIV)
HPYLSS1_01051	II	GTAC	Yes (RsaI)
HPYLSS1_01074	II	GCGC	Yes (HhaI)
HPYLSS1_01310	II	GANTC	Yes (HinfI)
HPYLSS1_01326	III	Un	No
HPYLSS1_01339	III	Un	No
HPYLSS1_01406	II	Un	No
HPYLSS1_01436	II	CCATC	Yes (BccI)

HPYLSS1_01457	III	Un	No
HPYLSS1_01493	II	TGCA	Yes (HpyCH4V)

Table 2. Predicted Restriction Enzymes in SS1/PMSS1 genome. Data taken from RE-Methylases-All-SS1-Table_Jan30. Un = Unknown restriction site. Geneious column indicates they were included in the Geneious restriction enzymes file that was used to identify potential sites. * indicates this sequence was also an under-represented Kmer.

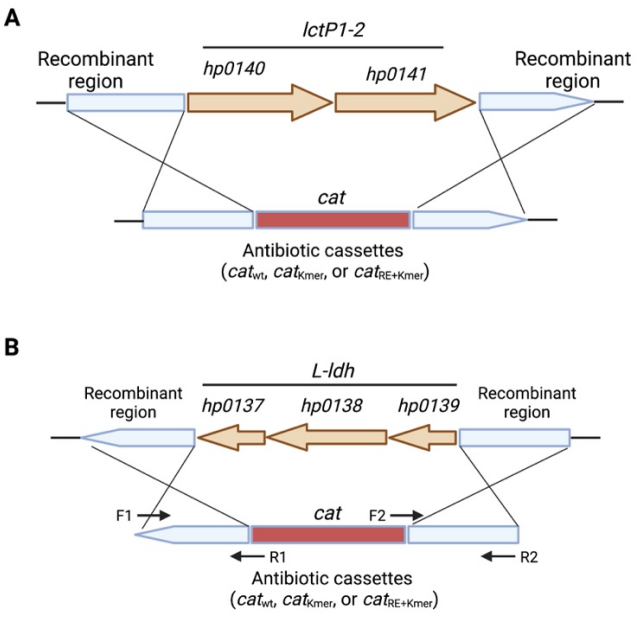
Table 4.3 Strains used in this study

Strain	Genotype or description	Reference and/or source(s)
H. pylori PMSS1	WT strain	Arnold et al., 2011
Campylobacter coli	WT *cat gene*	Wang et al., 1990
PMSS1 *ΔlctP1-2:: cat*$_{wt}$	*Δhp0140-0141::cat*$_{wt}$	This study (KOXX)
PMSS1 *Δlctp1-2::cat*$_{Kmer}$	*Δhp0140-0141::cat*$_{Kmer}$	This study (KO1710)
PMSS1 *Δlctp1-2::cat*$_{RE+Kmer}$	*Δhp0140-0141:: cat*$_{RE+Kmer}$	This study (KOXX)
PMSS1 *ΔL-ldh:: cat*$_{wt}$	*Δhp0137-0139::cat*$_{wt}$	This study (KOXX)
PMSS1 *ΔL-ldh:: cat*$_{Kmer}$	*Δhp0137-0139::cat*$_{Kmer}$	This study (KOXX)
PMSS1 *ΔL-ldh:: cat*$_{RE+Kmer}$	*Δhp0137-0139::cat*$_{RE+Kmer}$	This study (KOXX)
E. coli DH10B	#3019I	NEB
DH10B pUC59-*cat*$_{wt}$	*Δhp0140-0141::cat*$_{wt}$	This study (KOXX)
DH10B pUC59-*cat*$_{Kmer}$	*Δhp0140-0141::cat*$_{Kmer}$	This study (KOXX)
DH10B pUC59-*cat*$_{RE+Kmer}$	*Δhp0140-0141:: cat*$_{RE+Kmer}$	This study (KOXX)

Table 4.4 Primer set for generation of *L-ldh-cat* cassettes

Primer name	Sequence
Primer 1	TTAGTAGAGAATGATAGCGACTTTTTGAGGC
Primer 2	TCTGCCGAGAGTAGTGCGTCCTGCGGAATTGAAGCGATGCGTGAAGAGTTGTTT
Primer 3	ATTATATCATAAATCTATCCACTATATCATACATGTTGAATTCCGCATGCCCTTCAAACAA
Primer 4	TTGAAAGTCAATTTCTTTGCTACTTGTCT

Supplemental Figure



Supplemental Figure 4.1 Schematic of recombinant transformation based gene deletion in *H. pylori*

(A) Schematic of *H. pylori lctP1-2* gene deletion through the transformation with *cat* resistant cassettes contained plasmid. pUC59-cat_{wt}, pUC59-cat_{Kmer}, or pUC59-$cat_{RE+Kmer}$ plasmids were transformed to *H. pylori* WT, respectively. (B) Schematic of *H. pylori L-ldh* gene deletion. PCR amplification with primer set F1+R1 is for upstream region of *hp0137*; F2+R1 is for isogenic *cat* genes; and F2+R2 is for downstream region of *hp0139*. Three DNA fragments were annealed by using primer set F2 and R1. For both *lctP1-2* and *L-ldh* targeted transformation, equal amount of antibiotic caseates were introduced to WT *H. pylori* with either plate-based or liquid-based method.

4.6 Reference

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