Analysis of the roles of Helicobacter pylori lactate utilization genes in lactate-supplemented growth and bacterial pathogenesis

UNIVERSITY OF CALIFORNIA
SANTA CRUZ

A thesis submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in

MICROBIOLOGY AND ENVIRONMENTAL TOXICOLOGY

by

Cande Bernal

June 2021

The Thesis of Cande Bernal is approved:

_____
Professor Karen Ottemann

_____
Professor Vicki Auerbuch Stone

_____
Professor Chad Saltikov

_____
Quentin Williams
Acting Vice Provost and Dean of Graduate Studies

Table of Contents

Page

List of Tables

List of Figures

Figure **Page**

Supplemental Figures

Analysis of the roles of *Helicobacter pylori* lactate utilization genes in lactate-supplemented growth and bacterial pathogenesis
by
Cande Bernal

ABSTRACT

Helicobacter pylori is a human-adapted pathogen that colonizes the stomach and infects approximately half the world's population. *H. pylori* infection can induce erosive gastritis, peptic ulcers, and gastric cancer. *H. pylori* growth requirements in vivo are not yet fully understood, but one important molecule may be lactate. Lactate metabolism fulfills critical roles in mammalian and microbial homeostasis, through regulating immunological responses or serving as a precursor for various major microbial metabolites. Lactate exists in two enantiomers with distinct biological functions, L & D. L-lactate utilization in mammals and *H. pylori* has been studied more intensely relative to D-lactate metabolism and function. However, the physiological significance of mammalian D-lactate metabolism has become increasingly evident over the last decade. Recent findings such as the functional role of human D-LDH and the presence of neurotoxicity at physiological levels of D-lactate have contributed to the revaluation of mammalian D-lactate.

In this work, we performed in vitro temporal chemotaxis assays to assess *H. pylori* 's chemotactic response to both lactate isomers. We also created isogenic lactate uptake and metabolism mutants to assess the roles of these genes in lactate metabolism and stomach infection. Lactate supplementation growth assays were performed on lactate utilization mutants under various lactate concentrations, isomers and media compositions. In vivo murine infections with isogenic PMSS1 lactate metabolism mutants were performed to examine the significance of each lactate permease in gastric colonization. Here we report that D-lactate is a

chemoattractant sensed at physiological concentrations by the TlpC chemoreceptor in a manner distinct from L-lactate. We also found that the absence of *H. pylori* lactate permeases alters in vitro growth response and significantly reduces in vivo gastric colonization. These findings suggest that lactate utilization confers a functional role in *H. pylori* growth and colonization.

ACKNOWLEDGEMENTS

I am honored to have been bestowed a strong interdisciplinary graduate education under the guidance of the faculty and students in the UCSC Microbiology and Environmental Toxicology department. This dissertation only came to fruition via the contributive efforts made by my patient and considerate departmental colleagues. I thank my family, especially Olga Alicia Barrera & Carmen Romo, for the funds & fun they've graciously provided throughout my education.

I am indebted to various queer comrades who throughout my life have enriched my soul by providing me rarefied company, including my principal investigator Karen Ottemann and my dear friend Juliana Nzongo, since all of you have exhibited immeasurable influence in my attainment of self-realization. I also extend my gratitude towards my sister, Dayanara Bernal, for providing me unwavering support and many laughs throughout graduate school. In addition, I dedicate this thesis to my impeccable hippo hero Henry Versace, the best odorous boy-canine since time immemorial.

Microbiological Elegance

1. Introduction

1.1 Background

1.1.1 *Helicobacter pylori* as a chemotactic gastric pathogen

H. pylori is a motile microaerophilic Gram-negative bacterium that exclusively colonizes the human stomach, specifically the mucous layer and gastric glands (Bury-Moné et al. 2006; Jäger et al. 2000; Rolig et al. 2012; Schreiber et al. 2004; Sigal et al. 2015). A critical *H. pylori* colonization factor is chemotaxis. Chemotaxis refers to the modification of an organism's movement based off the chemical stimuli it receives through its chemoreceptors. The chemical stimuli can either induce a chemoattractant (flagellar run) or chemorepellant response (flagellar tumble). This change in the organism's movement is intended to reduce the organism's stress (Wadhams and Armitage 2004).

Chemotaxis is vital for the ability of *H. pylori* to swim through highly acidic regions of the stomach lumen and properly localize at its colonization sites. *H. pylori* has four distinct chemoreceptors, known as Transducer like proteins (Tlp) TlpA ,B ,C and D. Each detect distinct molecules such as urea, auto-inducer 2, and arginine (Keilberg and Ottemann 2016). However, multiple chemoreceptors can also detect the same molecule and collectively modify *H. pylori* chemotaxis. For instance, TlpA and TlpD were found to be independent acid sensors with TlpB dampening the TlpD-mediated chemorepellant response to HCl (Huang et al. 2017).

Although TlpC mutants have colonization defects in a co-infection model, the role of TlpC in the physiology of *H. pylori* remains largely unclear (Andermann, Chen, and Ottemann 2002; Machuca et al. 2017) The only known ligand detected by TlpC

is L-lactate (Machuca et al. 2017). *H. pylori* lactate utilization genes were identified based on their homology to *E. coli* lactate metabolism genes (Dong et al. 1993; Iwatani et al. 2014; Tomb et al. 1997). *H. pylori* encodes one L-lactate dehydrogenase (LDH), but this enzyme is not thought to actually produce L-lactate, and instead catalyzes the reverse reaction to oxidize lactate into pyruvate (Dong et al. 1993; Iwatani et al. 2014; Tomb et al. 1997). In *H. pylori*, pyruvate is a critical intermediate metabolite used to power the Kreb's cycle (Mendz, Hazell, and van Gorkom 1994).

1.1.2 Microbial L-lactate metabolism

Generally, L-lactate is produced by most microbial and mammalian cells during glycolysis, by conversion of pyruvate into lactate via LDH (Y.-S. Lee et al. 2018; Louis, Hold, and Flint 2014). Lactate formation occurs under anaerobic conditions to replenish NAD^+ for energy production via glycolysis (Akram 2013). This idea is an oversimplification, however, as many mammalian cells produce L-Lactate under aerobic conditions (Heinová, Kostecká, and Petrovová 2018). The typical human serum concentration of L-lactate is approximately 2mM. However, a variety of physiological situations can mildly or drastically increase the serum L-lactate concentration to as high as 40 mM (Walenta and Mueller-Klieser 2004).

L-lactate produced from cultured murine gastric surface mucus cells was observed to enhance *H. pylori* growth (Takahashi et al. 2007). *H. pylori* lactate utilization genes are conserved across almost all strains but not all strains exhibit the ability to catabolize lactate (W. C. Lee et al. 2017). *H. pylori* lactate genes were identified by Iwatani et al. 2014 through in vitro studies that assessed the contribution of these genes to growth, lactate uptake, and LDH activity. *H. pylori* encodes two

lactate permeases (LctP1 and LctP2), which is atypical since other *Helicobacter* species have only been observed to contain a single *lctP* gene. *H. pylori lctP1* expression is partially decreased by L-lactate but not D-lactate (Iwatani et al. 2014). For these analyses, *H. pylori* was grown in BB10 media supplemented with 5 mM or 10mM L-lactate. Expression levels of the *lctp1* gene were determined via qRT-PCR. There was a significant reduction in *lctp1* expression whereas the same concentrations of D-lactate did not alter *lctp1* expression. These results thus suggest that *lctp1* gene expression is regulated by L-lactate. *lctp2* has not yet been tested.

H. pylori also possesses a single L-LDH and D-LDH, which do not produce lactate (Chang et al. 1995; Iwatani et al. 2014). *H. pylori* has a total of four genes encoding LDH subunits: L-LDH is encoded by a three-gene cluster (HP0137-HP0139) that functions as a single unit while D-LDH is encoded by a single gene (HP1222) (Iwatani et al., 2014). LDH enzymes catalyze oxidation-reduction reactions and depend on electron carriers. In some LDH enzymes, electrons are carried on NAD+/NADH. In *H. pylori* strain 26695, L-LDH requires NAD+, while D-LDH is NAD+ independent (Iwatani et al., 2014). The only known physiological role of *H. pylori* LDH is the in vitro utilization of L-lactate as a carbon and energy source. Similarly, *Campylobacter jejuni,* a close relative of *H. pylori,* has also been observed to solely utilize L & D-lactate as a carbon/energy source as well (Thomas et al. 2011). *H. pylori* D & L-LDH were confirmed via qRT-PCR to be constitutively expressed when grown in BB media supplemented with 5 mM and 10mM of D-lactate and/or L-lactate (Iwatani et al., 2014). No known physiological conditions have been observed to modify *H. pylori* LDH activity.

Vertebrates contain six L-LDH isoenzymes, and these are all capable of producing L-lactate (Cristescu et al. 2008; Heinová, Kostecká, and Petrovová 2018). L-lactate confers a multitude of cellular signaling roles in mammals dependent on its serum concentration and localization. Examples include immune cell effector functions, wound healing, and vaginal mucosal immunity (Aldunate et al. 2015; Sun et al. 2017). Various pathogenic microbes depend on lactate genes to colonize mammalian hosts (Exley et al. 2005; Jiang et al. 2014; Lichtenegger et al. 2014). Lactate utilization-induced pathogenesis varies from biofilm development, complement resistance, and ROS resistance (Jiang et al. 2014; Sigurlásdóttir et al. 2019) L-lactate is a major carbon source in mucosal environments colonized by members of the *Lactobacillales* genus, such as *Streptococcus* and *Enterococcus,* which are found in the human stomach (Schoen et al. 2014; Schulz et al. 2016).

1.1.3 Microbial D-lactate metabolism

D-lactate in mammalian systems is produced via the intestinal microbiota and methylglyoxal detoxification, a ubiquitous & vital biological process(Louis, Hold, and Flint 2014; Thornalley 1990). Humans and *H. pylori* both encode a single D-LDH enzyme whose physiological role has remained largely unknown (Flick and Konieczny 2002; Iwatani et al. 2014; Monroe et al. 2019). In humans, D-lactate is largely produced and rapidly consumed by intestinal commensal microbes to fuel various metabolic pathways, such as the production of propionate, sulphide, or butyrate (Louis, Hold, and Flint 2014). Within mammals, D-lactate is either converted into pyruvate or quickly excreted (Ewaschuk, Naylor, and Zello 2005).

D-lactate is also produced by the glyoxalase system, which is observed in most cellular organisms (Thornalley 1993). The glyoxalase system converts

methylglyoxal, a toxic glycolytic by-product whose presence is ubiquitous amongst all cellular organisms, into D-lactate. (Silva et al. 2013). Methylglyoxal has been suggested to inhibit *H. pylori* (Keenan et al. 2010; Mavric et al. 2008; Takaji et al. 1997). *H. pylori* does not have any identified glyoxalase enzymes and lacks one of the characteristic enzymes of a glycolytic bypass, an alternate biochemical pathway that converts 2 glyceraldehyde 3-phosphate (GAP) into D-lactate (Pitson et al. 1999; Schilling et al. 2002; Thornalley 1993). Converting GAP directly into D-lactate lowers the possibility of GAP converting into an enediol intermediate and degrading into methylglyoxal. Therefore, it's unknown how *H. pylori* prevents methylglyoxal toxicity, which results in the inhibition of glycolytic enzyme activity via methylglyoxal irreversibly binding to proteins (Thornalley 1993).

Similarly to L-lactate, *H. pylori* does not produce D-Lactate and instead only catalyzes the reaction that oxidizes D-lactate into pyruvate (Chang et al. 1995). *H. pylori* D-LDH is NAD- independent and is structurally more similar to D-LDH encoded by *Pseudomonas putida* KT2440 rather than *E. coli* (Jiang et al. 2017; Tomb et al. 1997). These enzymes deposit the electrons from lactate directly into the electron transport chain. Mitochondrial human and murine D-LDH are widely expressed, predominantly in the kidney and liver (Flick and Konieczny 2002). However, D-lactate has historically been regarded as physiologically irrelevant to mammalian physiology due to its small serum concentration of approximately 70nM largely maintained through its rapid metabolic clearance. D-lactic acidosis occurs at serum concentrations exceeding 3mM, whose clinical symptoms include reversible neurological anomalies such as hallucinations and slurred speech. L-lactate does not appear to be neurotoxic (Ewaschuk, Naylor, and Zello 2005).

Recent evidence suggests that D-lactic acidosis is perhaps more common and grossly underreported, suggesting a potential increase in the physiological relevance of D-lactate in mammalian pathogenesis (Bianchetti et al. 2018; Monroe et al. 2019; Parrish 2015). Interestingly, there has been increasing evidence that links heightened mammalian D-LDH activity with the development of *H. pylori*-derived gastric adenocarcinomas (Kim et al. 2014; Parsons et al. 2017).The direct role of D-lactate in *H. pylori* metabolism and pathogenesis is currently unknown.

1.1.4 Gastric cancer induction via alterations to host metabolism

H. pylori is classified as a class 1 carcinogen and is the strongest risk factor for gastric cancer (F. Wang et al. 2014). Gastric adenocarcinomas have a high mortality rate due to their detection at typically advanced stages (Cormedi et al. 2018; Hohenberger and Gretschel 2003; B. R. Smith and Stabile 2009). It is well established that *H. pylori* is linked to gastric cancer through inducing the host's inflammatory cellular stress responses (F. Wang et al. 2014). It has been previously speculated that abnormal mitochondrial metabolism is the primary cause of cancer rather than uncontrolled cell growth (Akram 2013).

H. pylori has recently been evidenced to directly induce mitochondrial dysfunction and metabolic reprogramming (D. Y. Lee et al. 2017; Luo et al. 2016). CagA is a *H. pylori* virulence factor that is injected into gastric epithelial cells via a type IV secretion system (T4SS) and alters host signaling pathways and gene expression (Censini et al. 1996; Xiang et al. 1995). CagA $^{+}$ *H. pylori* strains, such as the PMSS1 strain used in this study, are associated with the development of and have been observed to be directly involved in the metabolic reprogramming of gastric

epithelial cells through various disruptions of host cell signaling (D. Y. Lee et al. 2017).

Gastric cancer cells are also well known to have metabolic alterations that create high levels of lactate. The Warburg effect in gastric cancer cells is canonically characterized by the metabolic shift away from oxidative phosphorylation towards lactate production under aerobic conditions (Liu et al. 2019). Additionally, heightened uptake of glucose and enhanced glycolysis also occurs in the Warburg effect (Liu et al. 2019). Heightened mammalian LDH activity has been evidenced to be correlated with cancer morbidity (Fantin, St-Pierre, and Leder 2006; Kim et al. 2014; Vinasco et al. 2019) *H. pylori* infected gastric biopsies were shown to have increased LDH expression as a result of increased oxidative stress (Baek et al. 2004) Gastric cancer tissue has also been observed to have significantly higher glyoxalase enzymatic activity, specifically GLO1, relative to healthy tissue (Hosoda et al. 2015; Thornalley 1990; Y. Wang et al. 2012). This increase in GLO1 is predicted to be caused by heightened glycolytic enzyme activity, although this measurement has not been directly reported. GLO1 was suggested to be a metabolic oncogene in human gastric cancer since its overexpression exhibited tumor-formation in vivo and GLO1 inhibition resulted in significantly reduced growth in a multitude of gastric cancer cell lines (Hosoda et al. 2015).

As mentioned earlier, *H. pylori* can utilize L and D lactate as a carbon/energy source to enhance growth (Iwatani et al. 2014; Takahashi et al. 2007). *H. pylori* encodes two lactate permeases (*lctP)* that have been observed to uptake L&D-lactate (Iwatani et al. 2014). Several pathogenic microbes encounter colonization defects upon disruption of lactate utilization genes (Jiang et al. 2014). Therefore, we

hypothesize that host-derived lactate has a role in *H. pylori* pathogenesis, especially during gastric cancer.

1.2 Justification for this research

The aim of this research was to elucidate the pathogenic role conferred by the *H. pylori* lactate metabolism genes, with an emphasis on microbial D-lactate metabolism since this metabolite has mostly been investigated in the context of intestinal microbiota. Defining the distinct chemotactic behavior that varying concentrations of L-& D-lactate exhibit allows us to better hypothesize the chemotactic behavior of *H. pylori* within the host's stomach. Characterizing the growth effect of lactate utilization gene mutants in the presence of lactate supplementation provides a deeper understanding of *H. pylori* physiology and serves as a foundation for the further investigation of bacterial lactate metabolism. The murine infections grant critical insight into the pathogenic significance of H. *pylori* lactate utilization genes. Cumulatively, the data arising from these experiments contributes foundational knowledge regarding the significance of microbial lactate metabolism within carcinogenic host-pathogen interactions.

2. Materials and methods:

Microbiology and molecular biology

2.1 Bacterial strains, solid and liquid media

The bacterial strains and plasmids used in this study are shown in Table 1. *H. pylori* strains were grown on Columbia Horse Blood Agar (CHBA) plates containing 50 µg/ml ß-cyclodextrin (VWR), 5ml/100ml agar defibrinated horse blood (Hemostat labs),10 µg/ml vancomycin, 5 µg/ml cefsulodin, and 2.5 U/ml polymyxin B (Gold

Biotechnology). For chloramphenicol blood plates, from a stock of 20 mg/ml, 75 µl/100ml agar was used. For kanamycin plates, from a stock of 30 mg/ml, 50 µl/100ml agar was used. For murine output plates, 75 µl/100ml agar of each of the following components were added; trimethoprim (5 µg/ml) / amphotericin B (8 µg/ml), bacitracin (200 mg/ml), and nalidixic acid (10mg/ml).

Cultures were grown in microaerobic conditions (5% O_2 & 10% CO_2) at 37°C. *H. pylori* liquid cultures were grown with constant shaking under microaerobic conditions in media denoted as BB10, consisting of brucella broth (BD BBL/ Fisher) containing 10% heat-inactivated fetal bovine serum (Life Technologies).

2.2 Construction of *H. pylori* mutants

The *H. pylori* PMSS1 and G27 Δ*lctP*1 & Δ*lctP*2 clean deletions were created via natural transformation of the wild type with plasmid pUC57-lctp1::cat & pUC57-lctp2::cat. Transformants were selected on chloramphenicol, and the mutations confirmed by PCR analysis of the mutated locus with the same primers used to generate the deletions (Table 2). WT and the lactate permease deletion strains had distinctly different band sizes, where the unaffected permease had a band size of 1.6kb and the mutant *lctP* had a band size of 1kb. G27 lactate permease deletions were confirmed by sequencing the PCR product. After verification, the strains were made GFP-positive by transforming with the plasmid pTM115 derived from PMSS1 and selecting for kanamycin resistance. Strains were confirmed to retain robust motility via phase-contrast microscopy.

Mutation of the *D-ldh* locus was done via natural transformation with a SOE PCR product containing a chloramphenicol cassette insertion. In brief, the 5’ and 3’ ends of G27 *D-ldh* were amplified in 20 µl PCR reactions with 1 µl of WT G27 gDNA

as the template & two distinct primer sets listed in Table 2. The chloramphenicol cassette in the pS102 plasmid (Table 1) was amplified through the prior mentioned 20 µl PCR reaction. A 50 µl SOE reaction using primer sets 1 & 2 (Table 2) was performed through combining the three PCR products containing the 500bp G27 D-LDH 5' & 3' fragments and the chloramphenicol cassette. The following PCR program was used to perform the SOE PCR reaction: 94°C 3 min, (6X 94°C 30 sec, 50°C 1 min), add 1 µl phusion polymerase (Seth Rubin's lab), 94°C 1min, (30X 94°C 30 sec, 50°C 1 min, 72°C 5 min), 72°C 7 min). The SOE PCR product was then directly used for natural transformation with G27 WT. The Δ*D-LDH::cat* cassette in G27 Δ*D-LDH* was PCR amplified and the dirty PCR product was used to transform PMSS1 WT into PMSS1 Δ*D-LDH*#1. PMSS1 Δ*D-LDH*#2 was generated by using splicing by overlap extension (SOE) PCR with primers #3-6 that amplified the *D-ldh* chromosomal region and cat gene from plasmid pBS-cat (Ottemann et al., 2002). The gene deletion starts from 301bp downstream of the D-ldh start codon to 292bp upstream of the D-ldh stop codon, in which terminatorless cat gene is in the same transcriptional orientation of *D-ldh*.

Table 1. Bacterial strains and plasmids.

Strains or plasmids	**Characteristics**	**Source**
Helicobacter pylori		
PMSS1	*H. pylori* wild-type strain, parental strain for mutants	Arnold, et al. 2011 *Gastroenterology*
PMSS1-GFP	PMSS1 pTM115, Kan^r	This study

PMSS1 *ΔtlpC::cat*	null mutant lacking *tlpC*	Machuca, M. A. et al. 2017 *Scientific reports*
PMSS1 Δ*lctP::cat*	Null mutant lacking both PMSS1 lactate permeases, *lctP_1* and *lctP_2*, Cm^r	Hu and Ottemann, unpublished
PMSS1 Δ*lctP1::cat*	*lctP_1* disrupted derivative of PMSS1, Cm^r	This study
PMSS1Δ*lctP1*-GFP	PMSS1 Δ*lctP1::cat* pTM115 Cm^r & Kan^r	This study
PMSS1 *ΔlctP2::cat*	*lctP2* disrupted derivative of PMSS1, Cm^r	This study
PMSS1 Δ*lctP2*-GFP	PMSS1 *ΔlctP2::cat* pTM115 Cm^r & Kan^r	This study
PMSS1 *ΔD-LDH::cat*	*HPYLPMSS1_01172* disrupted derivative of PMSS1, Cm^r	This study
G27	*H. pylori* wild-type strain, parental strain for mutants	Baltrus, David A. et al. 2009 *Journal of bacteriology*
G27 Δ*lctp1::cat*	*HPG27_127* disrupted derivative G27, Cm^r	This study
G27 *Δlctp2::cat*	*HPG27_128* disrupted derivative of G27, Cm^r	This study
G27 *ΔD-LDH::cat*	*HPG27_1166* disrupted derivative of G27, Cm^r	This study

*E. coli DH5*α	Host strain used for plasmid construction	Silva, F., Lourenco et al. 2011 *Process biochemistry*
Plasmids		
pUC57	*E. coli* cloning vector, Amp^r	Shaffer, C. L et al. 2011 *PLoS pathogens*
pS101	PUC57 derivative with (HPG27_127)::*cat*	This study
pS102	PUC57 derivative with (HPG27_128)::*cat*	This study
pTM115	*ureAp*-GFP *aphA3*, Kan^r	Keilberg, D. et al. 2016 *mBio*
pBS-cat	pBluescript::*cat* (from *Campylobacter coli*), Amp^r , Cm^r	(Ottemann and Lowenthal 2002)

Kan^r, kanamycin resistance Cm^r, chloramphenicol resistance, Amp^r, ampicillin resistance.

Table 2. Primer sequences

Title	Primer sequence
Primer set #1 3' D-LDH fragment	Primer 1: 5' TTAAAGCGTGCAAGCATCCACTAA 3' Primer 3 CAT-F: 5'ATTATATCATAAATCTATCCACTATATCATA AAAAAAACCATGCGATTTTTTTA 3'

Primer set #2 5' D-LDH fragment	Primer 2: 5' GTGGAAGAAAATTATCATGCTTTTTTT 3' Primer 4 CAT-R: 5'TCTGCCGAGAGTAGTGCGTCCTGCGGATTTTGCTCCACCCCGCAACACAT 3'
Primer #3	5' TTAAAGCGTGCAAGCATCCACTAAATA 3'
Primer #4	5'ATTATATCATAAATCTATCCACTATATCATAACGATGTGCGCTTTAAAATTAGAAAATAAA 3'
Primer #5	5'TCTGCCGAGAGTAGTGCGTCCTGCGGATTGAGCGTCTTTGAAAAAATGTGCCAC 3'
Primer #6	5' CGGTAAAAAAAGCATGATAATTTTCTTCCAC 3'
Cm cassette	catF: 5'TATGATATAGTGGATAGATTTATGATATAAT catR: 5'TCCGCAGGACGCACTACTCTCGGCAGA
G27 *lctP1*	HPG27_127 FWD: 5' ATGGAATTTTATCAAGTCTATGA 3' HPG27_127 RVS: 5' TTAAGTAGGAGTAACTGGAATAATAAA 3'
G27 *lctP2*	HPG27_128 FWD: 5' GTGTCAGAATTTCATCAAGTTTAT 3' HPG27_128 RVS: 5' CTACTTTATCGCAGGCACCACTT 3'
PMSS1 *lctP1*	HPG27_127 FWD: 5' ATGGAATTTTATCAAGTCTATGA 3' 140 PMSS1 RVS: 5' TTATTTAGGAATGATAGGGATAATA 3'
PMSS1 *lctP2*	141 PMSS1 FWD: 5' GTGCTAGAATTTCATCAAATTTAT 3' HPG27_128 RVS: 5' CTACTTTATCGCAGGCACCACTT 3'

3. Materials and methods: Characterization of chemotaxis

3.1 Video microscopy analysis of chemotaxis

Temporal swimming behavior assays were performed as done previously in Machuca, M. A. et al. 2017, with PMSS1 WT and its isogenic Δ*tlpC* variant grown in 5mL BB10 with constant shaking under microaerobic conditions at 37°C for 12 hours to reach exponential growth phase. The cultures were diluted to an OD_{600} 0.1 in fresh BB10 and incubated as above for 40 minutes. Robustly motile cultures were treated with sodium L or D- lactate (0.1 mM, 1 mM, 10 mM) (Sigma-Aldrich). 50 μM dipyridyl treatment served as a chemoattractant control as used previously (Machuca et al. 2017) resulting in fewer direction changes. 10 mM HCL treatment was used as a chemorepellant control (Huang et al. 2017; Iwatani et al. 2014; Machuca et al. 2017) and untreated samples were used as controls for baseline swimming behavior. BB10 pH upon treatment was separately determined through the use of a Denver instruments pH meter. Cultures were filmed immediately after ligand addition at 400x magnification using a Hamamatsu Digital Camera C4742-95 with the μManager software (Version 1.4.22), mounted on a Nikon Eclipse E600 phase contrast microscope(Edelstein et al. 2014). For each sample, 200 3-s-long bacterial tracks from three independent cultures were analyzed manually to identify stops followed by direction changes and to calculate the average number of direction changes in 3 seconds. Statistical analysis of the data for treated versus untreated samples was performed using a Student's t-test.

4. Materials and methods: Liquid growth curves

4.1 Fluostar BMG plate reader

For liquid growth curves, several conditions were tested, including Brucella Broth and Ham's F-12 containing different percentages of FBS (10% and 2% respectively). All liquid growth curve cultures were grown to exponential phase (≈12 hrs) before being diluted to OD_{600} 0.1 in a 200µl culture within a 96-well plate for 36 hours. 1-2 µl of lactate supplementation was provided immediately prior to the initiation of the 36-hour growth experiment. Absorbance readings were performed every 20 minutes in a Fluostar BMG plate reader.

5. Materials and methods: In vivo murine infections

5.1 Infecting mice via pipette feeding & gastric colonization enumeration

For the initial infection, female C57BL/6 mice were 6-8 weeks old at the time of *H. pylori* infection. These mice were ordered from Charles River Labs. For the second infection, a mix of approximately 8-month-old female and male C57BL/6 mice were used, which had been bred in house. Animals were infected orally via a 200 µl pipette (Pipet-Lite) with 50 µl of PMSS1 WT-GFP, Δ*lctP*-GFP, Δ*lctP1*-GFP, and Δ*lctP2*-GFP grown to late exponential phase in BB10 media, then spun down and concentrated to OD_{600}=3. After the 2-week infection period, the animals were sacrificed via CO_2 narcosis and the stomach was removed by cutting at the stomach-esophageal junction and the antrum-duodenum sphincter. The fore stomach was removed, and the stomach opened along the lesser curvature. The stomach was gently rinsed in 25 ml ice-cold phosphate-buffered saline (PBS) to remove food.

Blood vessels and the muscle layer were removed from the nonluminal side of the tissue using microscissors (Kelly Scientific) and tweezers. The stomach was then dissected to isolate the corpus from the antrum using the difference in tissue coloration as a marker for the border between these regions. Each piece was then cut into two, one for total bacterial number and one for gland isolation. For total bacterial number, the tissue was weighed, homogenized using the Bullet Blender (Next Advance) with 1.0-mm zirconium silicate beads, and then plated to determine the number of CFU per gram of stomach tissue on murine output CHBA plates. To enumerate bacterial colonies, plates were analyzed using a Bio-Rad Chemi-Doc imaging system using settings for fluorescent antibodies at 488.

5.2 Gastric gland occupancy

Glands were isolated using a protocol adapted from (Keilberg et al. 2016; Mahe et al. 2013). Dissected gastric tissue was cut into 1-mm2 pieces and incubated with slight shaking in Dulbecco's phosphate-buffered saline (DPBS) (Millipore) plus 5 mM EDTA at 4°C for 2 h. After this period, the tissue was transferred into ice-cold DPBS containing 1% sucrose and 1.5% sorbitol and shaken roughly by hand for 2 min. The remaining large tissue pieces were allowed to settle, and 2 ml of the solution containing the glands was removed. Glands were then transferred into a clean tube, washed two times with 2 ml DPBS each time and labeled with 10 g/ml Hoechst DNA stain (Life Technologies). Glands were kept on ice until examined. Ten microliters of isolated glands that had been stained with Hoechst 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 individual

infection,100 glands each were imaged for the corpus and antrum, and the number of *H. pylori* cells inside the gland was counted manually for each gland.

6 Results

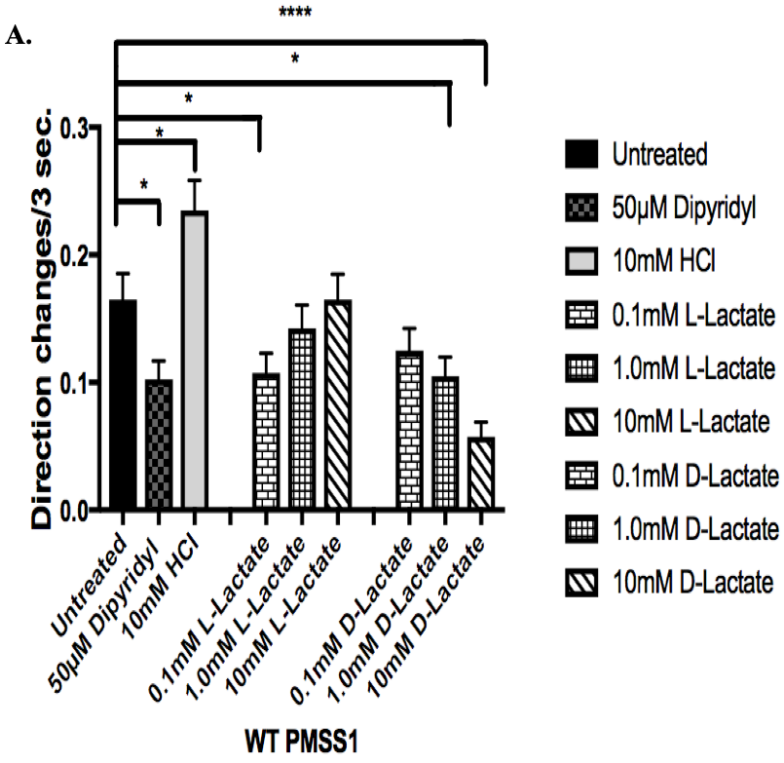
6.1 *H. pylori* TlpC senses D-lactate as a chemoattractant. The crystal structure of the ligand binding site for *H. pylori* chemoreceptor TlpC has been resolved and the L-lactate binding site was identified in this region (Machuca et al. 2017). Temporal chemotaxis assays confirmed L-lactate to be a chemoattractant sensed by TlpC (Machuca et al. 2017). Due to the chirality of lactate, we investigated whether D-lactate induced an appreciable chemotactic response in *H. pylori* PMSS1. PMSS1 has been used in recent chemotaxis studies and is a physiologically relevant strain due to encoding a complete cytotoxin-associated gene pathogenicity island (cagPAI) (Huang et al. 2017; Machuca et al. 2017) The *cag*PAI was detected in 90% of biopsies taken from patients who suffered severe gastric diseases and is an important *H. pylori* virulence factor (Stein, Rappuoli, and Covacci 2014).

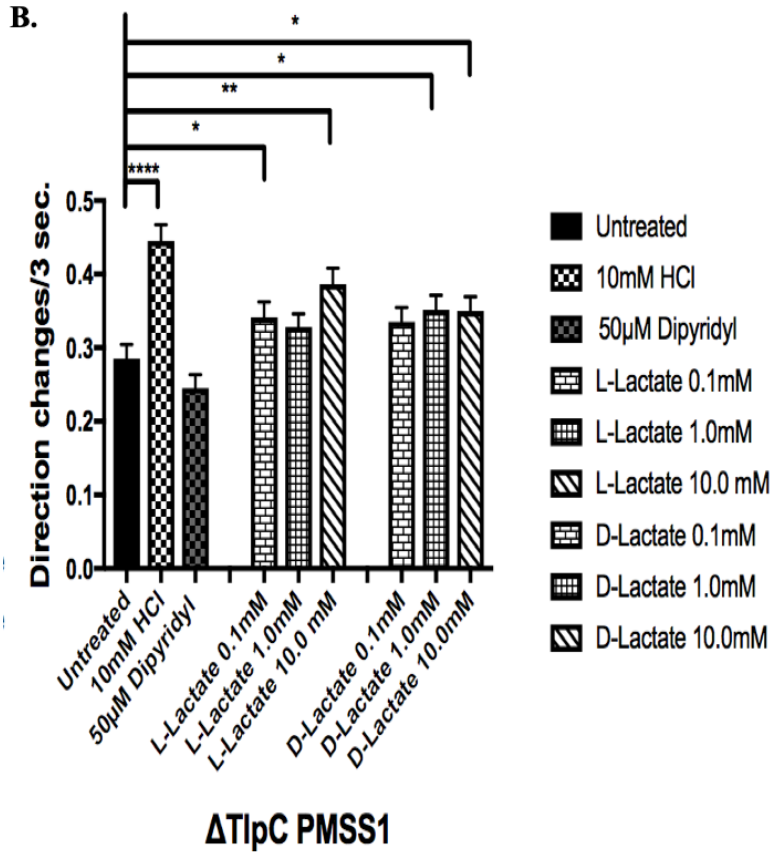
Temporal chemotaxis assays were performed as previously described, where chemorepellents and chemoattractants respectively induce increased and decreased direction changes (Machuca et al. 2017). HCl is a known chemorepellant that TlpA and TlpD senses via detection of low pH (Huang et al. 2017). Dipyridyl is a chemoattractant sensed by TlpD (Collins et al. 2016). WT PMSS1 showed a significant chemoattractant response to 10 and 1 mM but lost the response at 0.1mM D-lactate (Fig.1A). In accordance with the observations reported in Machuca et al. 2017, 0.1mM L-lactate induced a strong chemoattractant response whereas 10mM L-lactate did not significantly alter PMSS1 chemotaxis (Fig. 1A). Strikingly, the chemotactic response to identical concentrations of L& D-lactate are distinct. The

rich bacterial growth medium used in these chemotaxis assays, BB10, was analyzed via a pH probe and found to have negligible pH alterations (<0.2 pH) with L- or D-lactate supplementation.

A previously generated isogenic null mutant strain of TlpC (Δ*tlpC*) was used to determine whether D-lactate was specifically sensed by TlpC (Machuca et al. 2017). The chemoattractant response of L&D-lactate were abrogated in the Δ*tlpC* strain at all tested concentrations (Fig. 1B). These results suggest that TlpC is the chemoreceptor for L&D-lactate and that both lactate isomers induce chemoattractant responses at distinct concentrations.

Figure 1 Chemotaxis assay performed on *H. pylori* PMSS1 WT (1A) & ΔTlpC PMSS1 (1B) strain of *H. pylori.* Lactate induced a TlpC dependent chemoattractant response. Dipyridyl and HCl served as the attractant and repellant control, respectively. Error bars represent standard error of the mean. *p<0.05; **p<0.01,****p<0.0001, comparisons performed via two-way ANOVA. This data is representative of 3 biological replicates.


A.
****
*
*
*
*
*
Direction changes/3 sec.
0.0
0.1
0.2
0.3
Untreated
50μM Dipyridyl
10mM HCl
0.1mM L-Lactate
1.0mM L-Lactate
10mM L-Lactate
0.1mM D-Lactate
1.0mM D-Lactate
10mM D-Lactate
Untreated
50μM Dipyridyl
10mM HCl
0.1mM Lactate
1.0mM Lactate
10mM Lactate
0.1mM D-Lactate
1.0mM D-Lactate
10mM D-Lactate
WT PMSS1


B.
*
*
**
*
****
Direction changes/3 sec.
0.0
0.1
0.2
0.3
0.4
0.5
Untreated
10mM HCl
50μM Dipyridyl
L-Lactate 0.1mM
L-Lactate 1.0mM
L-Lactate 10.0 mM
D-Lactate 0.1mM
D-Lactate 1.0mM
D-Lactate 10.0mM
Untreated
10mM HCl
50μM Dipyridyl
L-Lactate 0.1mM
L-Lactate 1.0mM
L-Lactate 10.0 mM
D-Lactate 0.1mM
D-Lactate 1.0mM
D-Lactate 10.0mM
ΔTlpC PMSS1

6.2 *H. pylori* D-LDH has an inconclusive role in carbon source utilization. L-lactate metabolism enhances *H. pylori* growth in vitro in a L-LDH dependent manner (Iwatani et al. 2014; Takahashi et al. 2007). L-LDH is encoded by a three-gene cluster (HP0137-HP0139) that functions as a single unit while D-LDH is encoded by a single gene (HP1222) (Iwatani et al. 2014). Although the growth of *H. pylori* isogenic lactate utilization deletion mutants has been previously characterized with 10mM L&D-lactate supplementation in the 26695 strain, it has been suggested that 10mM L- or D-lactate could be cytotoxic to *H. pylori* (Iwatani et al. 2014; Takahashi et al. 2007). Therefore, we performed growth assays with varying lactate concentrations and media and used the mouse-infecting strain PMSS1, as well as its isogenic lactate utilization deletion mutants.

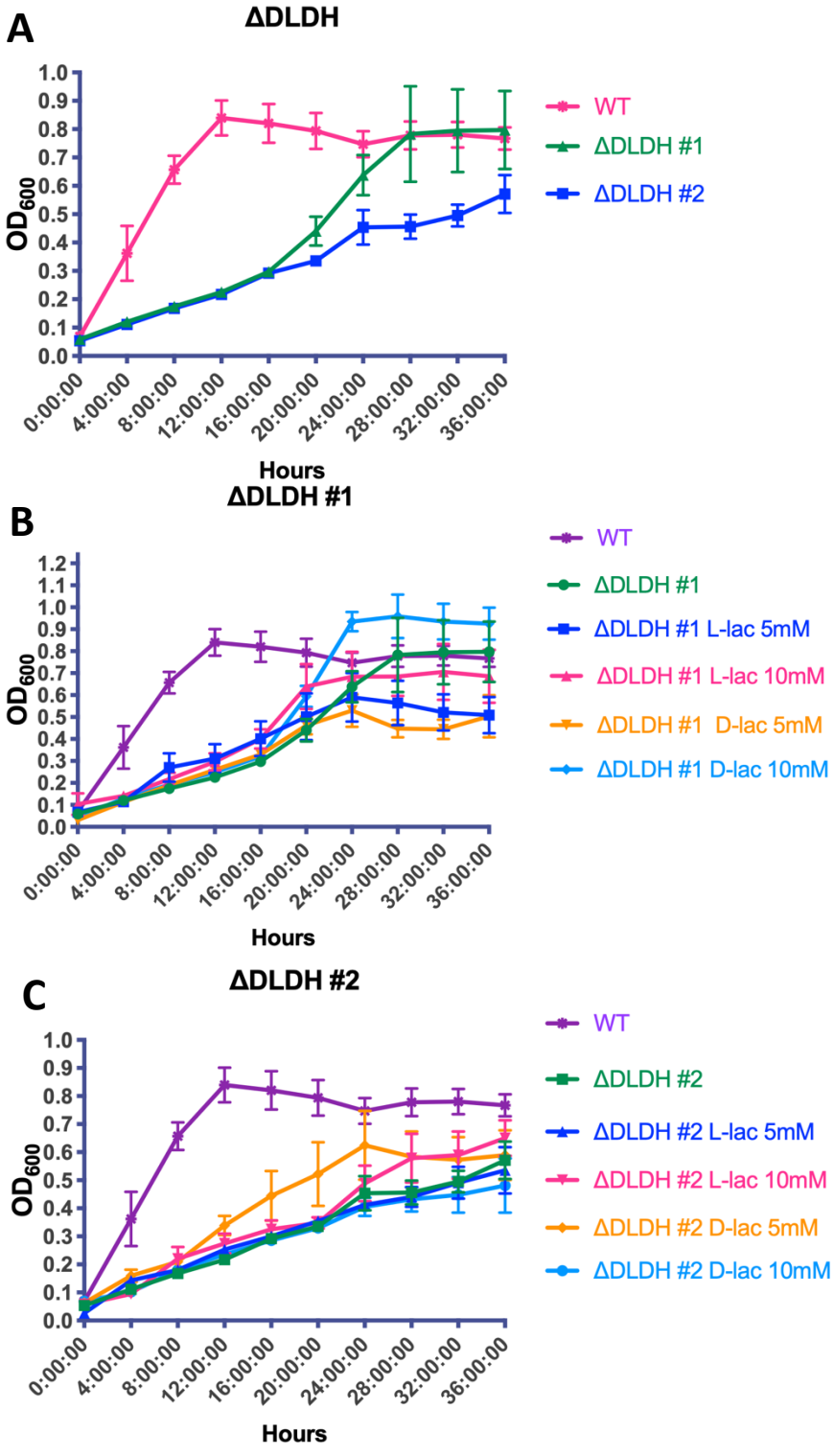
Two independently created PMSS1 ΔD-*ldh* mutants were generated to compare their growth to WT and gauge the metabolic role of *H. pylori* D-LDH. These mutants were generated by transforming PMSS1 with the same D-*ldh* allele that replaced all of the open reading frame with the cat gene. These transformants were confirmed by PCR amplification of D-*ldh* gene, showing the mutant allele had a size of 1.9 kb with 3' and 5' D-LDH fragment primers while the WT allele had a size of 2.8 kb.

We first confirmed that WT PMSS1 responded to D- and L-lactate. In nutrient rich BB10 media, PMSS1 WT demonstrated a slight growth advantage with 10mM D-lactate supplementation in between 12 and 16 hours, the exponential growth phase for this strain (Supplemental Figure 1A). Additionally, L-lactate supplementation increases PMSS1 WT growth rate in a modest but statistically significant manner (Hu and Ottemann, unpublished). These findings suggest that both D and L lactate

enhance *H. pylori* PMSS1 growth, similar to findings reported with strain 26695 (Iwatani et al. 2014).

We next characterized the behavior of the PMSS1 ΔD-*ldh* mutants. In the rich growth medium BB10 with no additional lactate, the variants initially grew slower than WT, but ΔD-LDH#1 recovered WT-levels of growth in stationary phase, but the other mutant, ΔD-LDH#2, remained low (Fig.2A). The reason for this variation is not yet known, so we analyzed both mutants for the response to lactate. The ΔD-LDH variants showed only a modest response to lactate supplementation that was non-identical. ΔD-LDH#1 exhibited enhanced growth with 10mM D-lactate supplementation but did not respond to L-lactate (Fig.2B). In contrast, the ΔD-LDH#2 mutant showed only a slight response to D-lactate, and generally only displayed poor growth under all conditions (Fig.2C). The previous analysis of *H. pylori* ΔD-LDH growth resembled what we see here for ΔD-LDH#2, where it was reported that 10mM L-lactate enhanced growth while 10mM D-lactate reduced growth (Iwatani et al. 2014). *Shewanella oneidensis* is a sedimentary metal-reducing facultative bacterium that possess a L-LDH and D-LDH structurally similar to that of *H. pylori* (Pinchuk et al. 2011; Pinchuk et al. 2009; Venkateswaran et al. 1999). Pinchuk et al. 2009 observed via mutagenesis that *S. oneidensis* L-LDH and D-LDH are required for the ability to grow on their respective lactate enantiomer in minimal media. Since both of the PMSS1 ΔD-LDH strains gained a slight a growth advantage with differing concentrations of D-lactate supplementations, further sequencing is required to conclude the validity of the ΔD-LDH mutants and the metabolic role of *H. pylori* D-LDH.

Figure 2 Liquid growth curves performed on *H. pylori* PMSS1 WT and ΔD-LDH variants. WT & ΔD-LDH with no lactate supplementation (2A), lactate supplementation of ΔD-LDH#1 (2B) and ΔD-LDH#2 (2C). *H. pylori* was grown to exponential phase in 5mL BB10 media shaking under microaerophilic conditions at 37ºC before being diluted to OD_{600} 0.1 in a 200µl culture within a 96-well plate for 36 hours. These growth curves were generated on 2 separate occasions with 3 technical replicates for each condition. Absorbance readings were performed every 20 minutes in a Fluostar BMG plate reader, for clarity, readings were only shown in 4-hour intervals. Error bars represent standard error of the mean and this data is representative of 2 biological replicates.

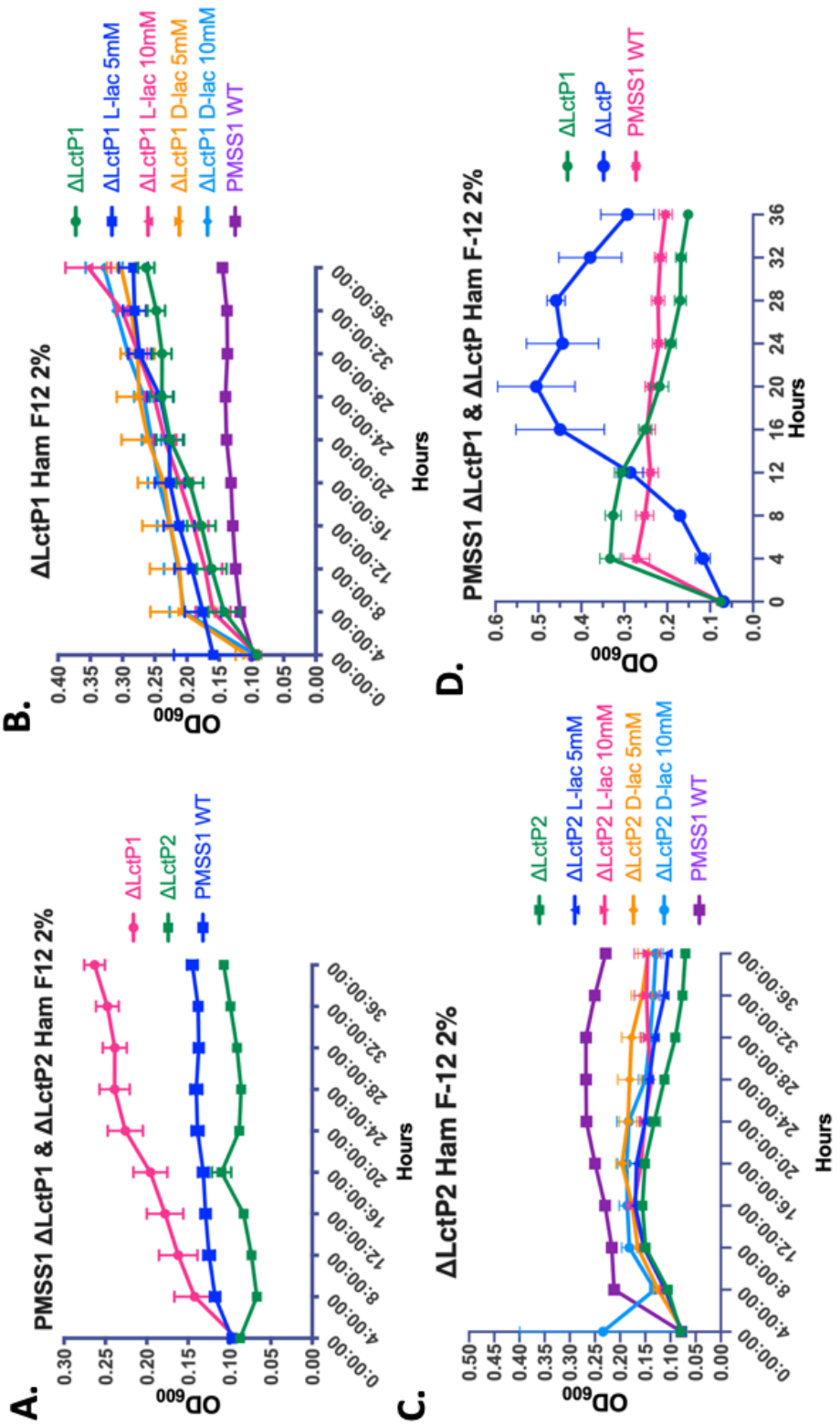

A
ΔDLDH
OD600
Hours
WT
ΔDLDH #1
ΔDLDH #2
B
ΔDLDH #1
OD600
Hours
WT
ΔDLDH #1
ΔDLDH #1 L-lac 5mM
ΔDLDH #1 L-lac 10mM
ΔDLDH #1 D-lac 5mM
ΔDLDH #1 D-lac 10mM
C
ΔDLDH #2
OD600
Hours
WT
ΔDLDH #2
ΔDLDH #2 L-lac 5mM
ΔDLDH #2 L-lac 10mM
ΔDLDH #2 D-lac 5mM
ΔDLDH #2 D-lac 10mM

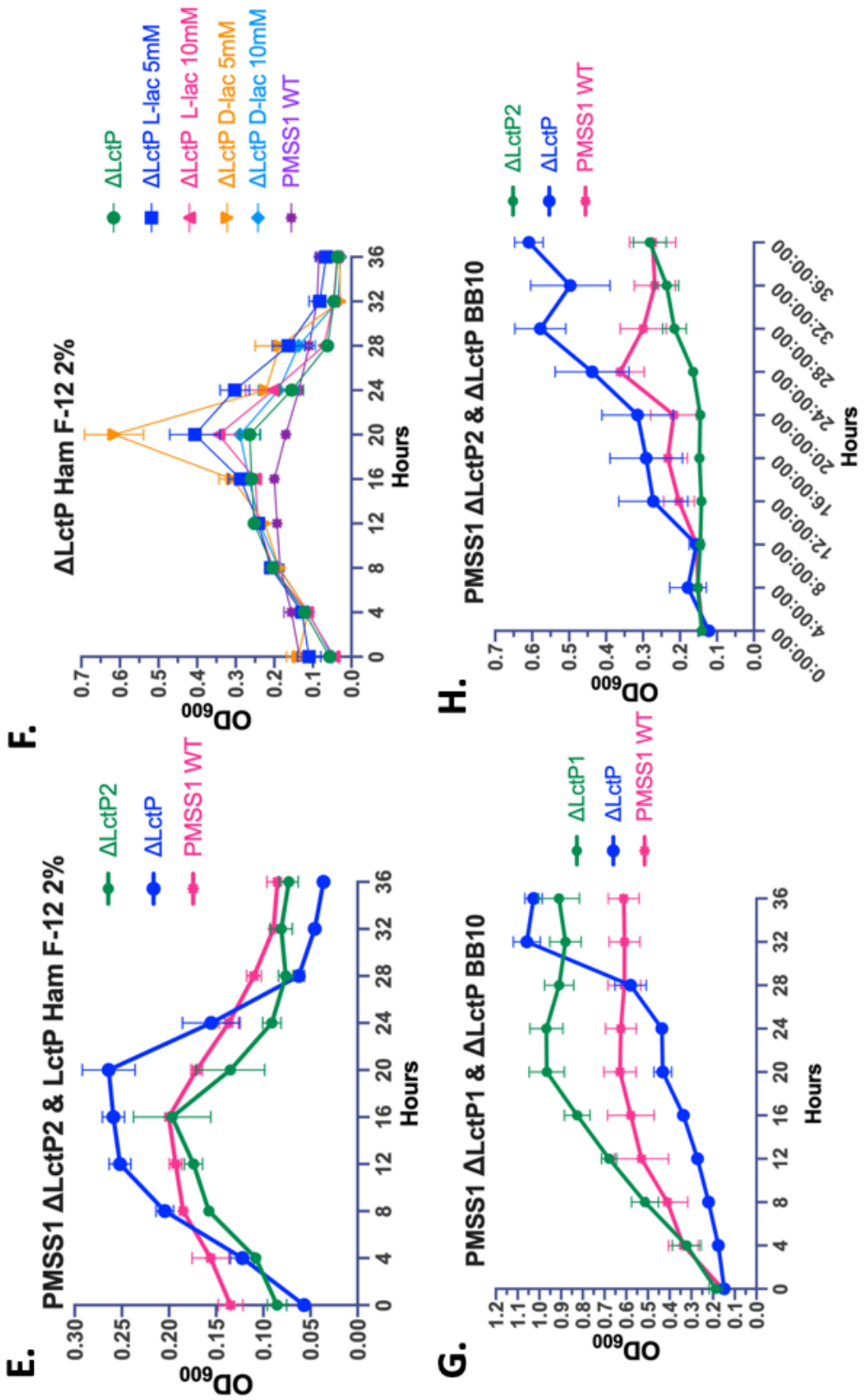
6.3 *H. pylori lctP* mutants exhibit enhanced liquid growth independent of lactate supplementation. *H. pylori* contains two distinct but similar lactate permease (Lct) genes (HP0140 & HP0141) responsible for transporting lactate. Although *H. pylori* has not had the biological characteristics of its putative lactate permeases characterized, HP0140 & HP0141 were identified due to their percent identities (respectively 58.7% & 55.5%) to *E. coli* lactate permease (LldP), which has been well studied (Tomb et al. 1997). Iwatani et al. 2014 reported that the *H. pylori* dual lactate permease mutant (Denoted as Δ*lctP* in that study) still had enhanced growth with L- and D-lactate supplementation in. Here we characterize the growth of individual lactate permease deletions (Denoted as *ΔlctP1* & *ΔlctP2*) and compare them to *ΔlctP* in a PMSS1 background.

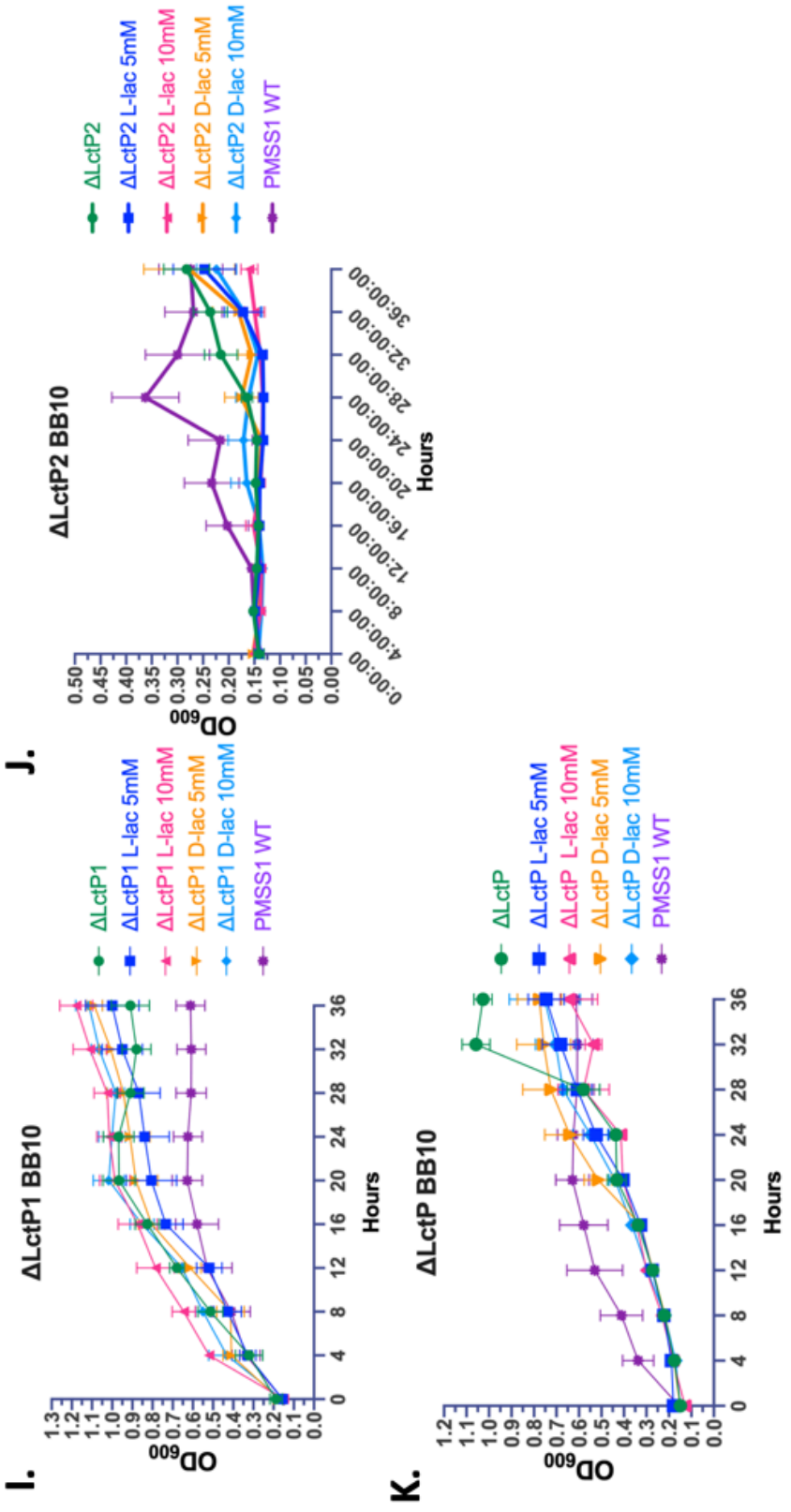
Hams F-12 is a defined medium that was used in this part of the study to simulate bacterial growth in limited nutrient conditions, and because we reasoned it would be easier to detect effects of lactate addition to this medium. All growth curve analysis performed in Hams F-12 were supplemented with 2% fetal bovine serum, rendering the media undefined. *H. pylori* PMSS1 *ΔlctP1* and *ΔlctP2* growth in Ham F-12 2% serum was directly compared to each other in four distinct growth curves. Multiple observations can be made from this data. First, WT PMSS1 grows very slowly in Hams F-12 2% serum, with an estimated doubling time of greater than 16 hours, compared to 4-12 hours in BB10 (Fig. 3) The *ΔlctP1* and double *lctP1-2* mutant generally grew better than WT, with doubling times of respectively 4-16 hours and 4-8 hours in Hams F-12 2% serum (Fig. 3A, 3D, 3E), while the *ΔlctP2* mutant comparatively grew to notably lower optical densities (Fig. 3A, 3C, 3E). Lactate supplementation generally had little effect on the growth of all lactate permease

mutants, with the exception of the double *lctP1-2* mutant, which had a notable increase in growth in the early log stage with 5mM of L or D lactate supplementation (Fig. 3B, 3C, 3F).

Figure 3 Liquid growth curves performed on *H. pylori* PMSS1 WT and Δ*lctP* variants. 3A-3F were measured in Ham F-12 2% serum. Δ*lctP1* & *lctP2* with no lactate supplementation (3A), *lctP1* lactate supplementation (3B), Δ*lctP2* lactate supplementation(3C), Δ*lctP1* & Δ*lctP* with no lactate supplementation (3D), Δ*lctP2* & Δ*lctP* with no lactate supplementation (3E), *lctP* lactate supplementation(3F). 3G-3K were measured in BB10. Δ*lctP1* & Δ*lctP* with no lactate supplementation (3G), Δ*lctP2* & Δ*lctP* with no lactate supplementation (3H), *lctP1* lactate supplementation (3I), Δ*lctP2* lactate supplementation(3J), *lctP* lactate supplementation(3K). *H. pylori* was grown to exponential phase in 5mL BB10 media shaking under microaerophilic conditions at 37ºC before being diluted to OD_{600} 0.1 in a 200µl culture of the denoted media within a 48-well plate for 36 hours. 5 technical replicates were used for each condition. Absorbance readings were performed every 20 minutes in a Fluostar BMG plate reader, for clarity, readings were only shown in 4-hour intervals. Error bars represent the standard error of the mean.


A.
PMSS1 ΔLctP1 & ΔLctP2 Ham F12 2%
ΔLctP1
ΔLctP2
PMSS1 WT
OD600
Hours
B.
ΔLctP1 Ham F12 2%
ΔLctP1
ΔLctP1 L-lac 5mM
ΔLctP1 L-lac 10mM
ΔLctP1 D-lac 5mM
ΔLctP1 D-lac 10mM
PMSS1 WT
OD600
Hours
C.
ΔLctP2 Ham F-12 2%
ΔLctP2
ΔLctP2 L-lac 5mM
ΔLctP2 L-lac 10mM
ΔLctP2 D-lac 5mM
ΔLctP2 D-lac 10mM
PMSS1 WT
OD600
Hours
D.
PMSS1 ΔLctP1 & ΔLctP Ham F-12 2%
ΔLctP1
ΔLctP
PMSS1 WT
OD600
Hours


E.
PMSS1 ΔLctP2 & LctP Ham F-12 2%
ΔLctP2
ΔLctP
PMSS1 WT
OD600
Hours
F.
ΔLctP Ham F-12 2%
ΔLctP
ΔLctP L-lac 5mM
ΔLctP L-lac 10mM
ΔLctP D-lac 5mM
ΔLctP D-lac 10mM
PMSS1 WT
OD600
Hours
G.
PMSS1 ΔLctP1 & ΔLctP BB10
ΔLctP1
ΔLctP
PMSS1 WT
OD600
Hours
H.
PMSS1 ΔLctP2 & ΔLctP BB10
ΔLctP2
ΔLctP
PMSS1 WT
OD600
Hours


I.
ΔLctP1 BB10
OD600
Hours
ΔLctP1
ΔLctP1 L-lac 5mM
ΔLctP1 L-lac 10mM
ΔLctP1 D-lac 5mM
ΔLctP1 D-lac 10mM
PMSS1 WT
J.
ΔLctP2 BB10
OD600
Hours
ΔLctP2
ΔLctP2 L-lac 5mM
ΔLctP2 L-lac 10mM
ΔLctP2 D-lac 5mM
ΔLctP2 D-lac 10mM
PMSS1 WT
K.
ΔLctP BB10
OD600
Hours
ΔLctP
ΔLctP L-lac 5mM
ΔLctP L-lac 10mM
ΔLctP D-lac 5mM
ΔLctP D-lac 10mM
PMSS1 WT

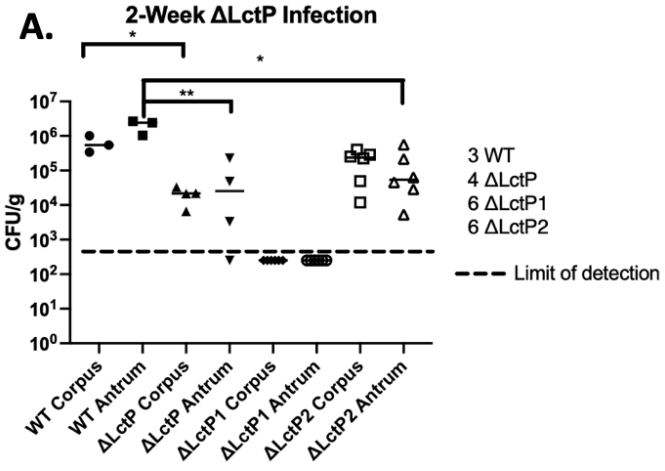
In nutrient rich media, some trends persisted. The *ΔlctP1* mutant still grew better than the WT (Fig. 3G). Generally, *ΔlctP1* grew the best of those analyzed based on the doubling times between 4 and 8 hours, followed by *ΔlctP*, then WT and finally *ΔlctP2* (Fig. 3G-3K). D or L lactate supplementation did not have a great or consistent effect with any of the mutant strains. Our lab has demonstrated that L-lactate supplementation provides a significant growth advantage to PMSS1, while D-lactate provides a slight growth advantage (Supplemental Figure 1 and unpublished data). In summary, these growth curves demonstrate that *ΔlctP1* and *ΔlctP* outgrow WT in nutrient rich or poor conditions and lactate supplementation slightly alters the growth of any lactate permease mutant.

6.4 *H. pylori* lactate permease genes promote colonization in vivo. The genes encoding the lactate permeases are the most conserved component in microbial lactate utilization operons (Jiang et al. 2014; Pinchuk et al. 2009). Additionally, for various pathogenic bacterial species, the single lactate permease has been observed to be necessary for successful in vivo colonization (Exley et al. 2007; Jiang et al. 2014; Lichtenegger et al. 2014). It was not known, however, whether both or one lactate permease would be needed in the case of microbes that encode two. We therefore attempted to elucidate the importance of both lactate permeases in *H. pylori* pathogenesis through infecting 6–8-week-old C57BL6/N female mice with variants of *H. pylori* PMSS1 for two weeks. Consistent with previous results, PMSS1 *ΔlctP* has a significant colonization defect in the antrum and corpus compared to WT (Fig. 4). PMSS1 *ΔlctP2* only had a significant colonization defect in the antrum, compared to WT, and not a defect in corpus colonization (Fig. 4). *ΔlctP1* was unable to establish a successful infection in either the corpus or the antrum, in two distinct in

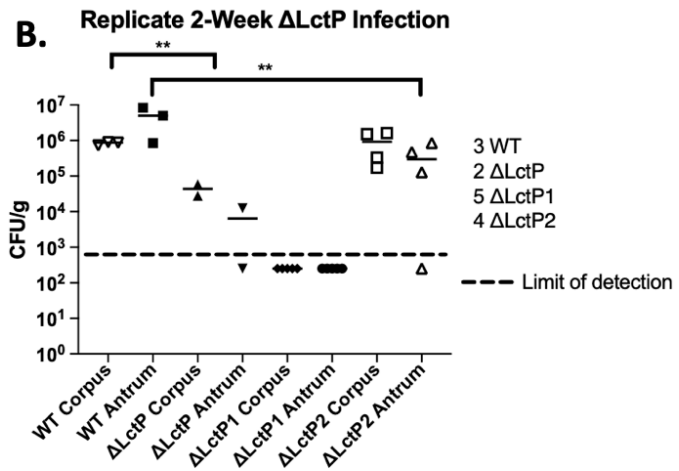
vivo challenges, resulting in no colony forming units on the mouse output plates (Fig. 4). This phenotype is curious considering that the dual LctP deletion was able to infect mice (Fig.4A-B).

The second in vivo challenge possessed several significant confounding factors, the mice were generally much older (= 8 months), some of the mice were male and three mice were pregnant. However, the phenotypes of the overall stomach colonization and gastric gland occupation exhibited similar phenotypes to what was observed in the initial infection challenge (4B-D). Overall, we observed that *lctP1* is required for establishing a successful gastric infection whereas *ΔlctP2* has a less severe colonization defect relative to *ΔlctP*.

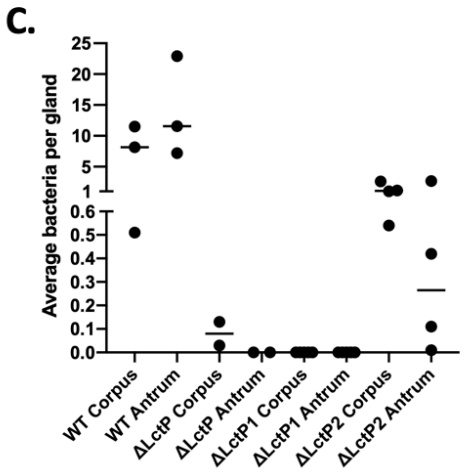
Figure 4 Two week in vivo murine infections with 6–8-week-old female C57BL6/N mice and fluorescently labeled variants of *H. pylori* PMSS1. Mice were infected with 50µl of a $3OD_{600}$ culture for 2 weeks before sacrifice via CO2 narcosis. Stomach tissue was removed, homogenized, and plated on CHBA plates to be enumerated (4A). In vivo infections were replicated in 8-month-old male and female C57BL6/N mice (4B). Gastric gland occupancy was characterized for replicate murine infection (4C-D). *p<0.05; **p<0.01, comparisons performed via student t-test.

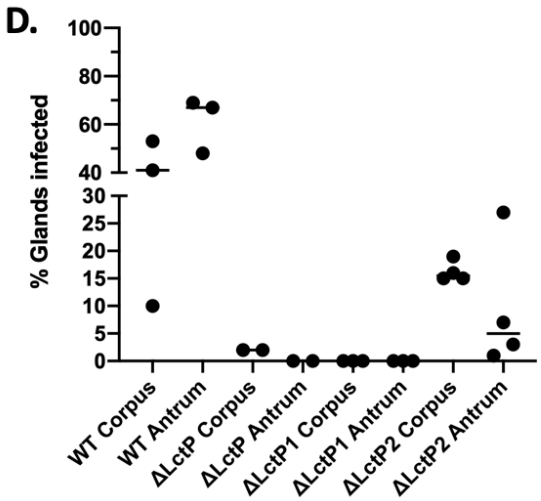


WT innoculation= 2.133E9 CFU/mL
ΔLctP innoculation = 7.2E8 CFU/mL
ΔLctP1 innoculation = 4.3E8 CFU/mL
ΔLctP2 innoculation = 2.18E9 CFU/mL



WT innoculation= 1.42E9 CFU/mL
ΔLctP innoculation =5.8E8 CFU/mL
ΔLctP1 innoculation = 9.8E7 CFU/mL
ΔLctP2 innoculation = 1.07E9 CFU/mL





7 Discussion

7.1 Physiological implications of *H pylori* lactate chemotaxis

The physiological function of *H. pylori* 's distinctly different chemoattractant response to L and D lactate has yet to be investigated (Fig. 1). Since both lactate enantiomers serve as a carbon source for *H. pylori*, it is possible that this is the only benefit derived from lactate chemotaxis (Iwatani et al. 2014; Takahashi et al. 2007). However, various infectious microbes rely on lactate to evade the host immune response and enhance colonization. For instance, *N. meningitidis* possess lactate mediated complement resistance that is reliant upon lactate catabolism to increase sialic acid biosynthesis, enabling sialyation & reducing C3 deposition on cell surface (R. M. Exley et al. 2005; Lo et al. 2009). Jiang et al. 2014 reviews additional mechanisms of lactate utilization induced microbial pathogenesis, including enhanced resistance to oxygen-dependent bactericidal mechanisms and nitrous oxide resistance. Overall, further investigation into lactate induced alterations of *H. pylori* physiology and metabolism could initiate the characterization of a novel *H. pylori* pathogenic mechanism.

I proposed a hypothesis that *H. pylori* could use its chemoattractant response to lactate as a means of enhancing gastric glandular colonization. *H. pylori* chemotaxis is necessary for maximum glandular colonization during the early infection phase (Keilberg et al. 2016). Leucine-rich repeat-containing G-protein coupled receptor 5-positive (Lgr5+) cells serve as stem/progenitor cells in the base of gastric glands and have been found to drive gastric adenocarcinoma formation (Barker et al. 2010; Phesse and Sansom 2017; Uehara et al. 2013; X. Wang et al. 2018; Xi et al. 2014). *H. pylori* colonizing murine and human gastric glands was

observed to directly interact and increase Lgr5+ stem cell proliferation in a manner heightened by the presence of *cagA* (Sigal et al. 2015). Additionally, chemotaxis deficient *H. pylori* strains had deficient gastric gland colonization and did not induce Lgr5+ stem cell proliferation (Keilberg et al. 2016; Sigal et al. 2015). Lgr 5+ cells are also found in the small intestine and colon, where they maintain small intestinal crypt homeostasis and are found to be highly expressed in colorectal adenocarcinomas (Baker et al. 2015; Rodríguez-Colman et al. 2017). Despite the hundreds of Lgr5+ stem cells present within murine adenomas, further studies observed a small amount of functional stem cells within individual murine intestinal crypts, suggesting that relatively few stem cells contribute to tumor growth (Humphries et al. 2013; Kozar et al. 2013; Schepers et al. 2012).

Intestinal Lgr5+ stem cell function is dependent upon surrounding lactate producing Paneth cells (Rodríguez-Colman et al. 2017). The L and D- lactate concentrations present in gastric mucosa and glands is unknown, rendering it difficult to predict the in vivo lactate-mediated chemotactic behavior of *H. pylori*. Additionally, it's unknown whether gastric Lgr5+ cells are functionally dependent upon lactate metabolism. However, the prior presented evidence (Refer to **1.1.4**) of *H. pylori* carcinogenic alterations to host metabolism and the promotion of mammalian LDH activity could suggest the presence of a heightened gastric lactate concentration.

Gastric cancer is a multifaceted disease, and it must be kept in mind that the genetics of the host and microbe have a critical role in determining the pathogenesis of *H. pylori* infection (El-Omar, Chow, and Rabkin 2001). *H. pylori* is genetically diverse, with bacteria from different geographic regions possessing variations in the presence and expression of virulence factors such as CagA and VacA (Akeel et al.

2019; Karlsson et al. 2012). The proteome expression pattern was also indicated to be geographically influenced since the protein expression pattern of several *H. pylori* strains was found to be dependent on their geographic habitat rather than their disease outcome or expression of *cagA* (Sugiyama et al. 2019) In addition, the proteome of *H. pylori* was suggested to be more diverse than the genome as evidenced by *H. pylori* from different regions in the stomach containing distinct patterns of protein expression despite a lack of genetic diversity (Ilina et al. 2010). Ultimately, the potential link between dysfunctional gastric Lgr5+ cellular activity and its influence on *H. pylori* chemotactic behavior warrants further studies.

7.2 *H pylori* respiratory D-LDH

D- and L-lactate as well as pyruvate have been evidenced to be readily oxidized by *H. pylori* (Kelly, Hughes, and Poole 2014). *H. pylori* L-LDH and D-LDH provide energy through oxidizing lactate into pyruvate. Pyruvate is metabolized via the TCA, serving as an immediate substrate for electron transport (H. Smith et al. 2001). The NAD-dependent and -independent LDH enzymes have different subcellular localizations. NAD$^+$ independent D-LDH is membrane-associated, while NAD-dependent L-LDH enzymes are cytosolic (Jiang et al. 2014, 2017). Previous work showed that *H. pylori* L-LDH was cytosolic, while D-LDH activity was in the membrane fractions (Chang et al. 1995).

Other organisms contain D-LDH that are predicted to be similar to that of *H. pylori*, and serve to enhance our understanding of the *H. pylori* enzyme. *Pseudomonas putida* KT2440 possesses a representative of microbial NAD$^+$ independent Fe-S D-iLDH, which has been well characterized biochemically and

structurally. This enzyme is of interest because it contains nearly identical domains as *H. pylori* D-LDH and has 59% protein similarity (Jiang et al. 2017). *Shewanella oneidensis* and *Campylobacter jejuni* both possess Fe-S D-iLDH similar to *P. putida* (Jiang et al. 2017; Pinchuk et al. 2009; Thomas et al. 2011). Here we analyzed two independent *H. pylori* PMSS1 D-LDH mutants. One (#2) appeared to have a severe growth defect, possibly suggesting it has another mutation in the chromosome. The other, ΔD-LDH#1, responded to high D-lactate supplementation with enhanced growth (Fig. 2). This finding contrasts with *S. oneidensis* ΔD-iLDH, which did not grow on D-lactate (Pinchuk et al. 2009). One possibility is that the remaining *H. pylori* "L"-LDH could be involved in D-lactate oxidation. This possibility has precedent: the expression of *S. oneidensis* L-LDH in a *E.coli* strain lacking D-LDH restored the mutant's ability to grow on D-lactate (Pinchuk et al. 2009). Additionally, previous work has shown that D-LDH's often oxidize L-lactate, as was observed in *C. jejuni* and suggested for *H. pylori*, because the ΔD-LDH strain was measured to have lower L-lactate oxidation activity relative to WT (Futai and Biol 1972; Iwatani et al. 2014; Jiang et al. 2017; Kato et al. 2010; Thomas et al. 2011) Due to the relatively early stage of microbial Fe-S D-LDH research and the variety of observed contrasting phenotypes, the *H. pylori* PMSS1 ΔD-LDH strains must be sequenced and the mutation complemented to conclusively determine the metabolic contribution of D-LDH.

7.3 *H pylori* lactate permeases

A few other microbes have been found that have more than one copy of *lctP*. *Desulfovibrio vulgaris* is an environmental anaerobic sulfate-reducing microbe and opportunistic pathogen that uses lactate as its primary energy source (Tang et al.

2007) *D. vulgaris* oxidizes lactate to eventually form acetate to serve as an electron donors for sulfate reduction. Most of its lactate is oxidized via respiratory membrane bound LDH enzymes. Multiple copies of *lctP* are present in the genome but only 2 were identified to be functional (Rajeev et al. 2019; Vita et al. 2015). One of the *D. vulgaris lctP* genes (DVU2451) was observed to have its expression dependent upon the presence of lactate (Rajeev et al. 2019). *H. pylori lctP1* expression was significantly reduced by L-lactate (Iwatani et al. 2014). Both functional *D. vulgaris* LctP proteins (DVU2451 & DVU3026) share 95% sequence similarity and were thought to be functionally redundant since deletion of DVU3026 increased DVU2451 expression (Vita et al. 2015). Whether deletion of one *H. pylori lctP* gene affects the expression of the other is not yet known.

We were surprised that the *H. pylori* PMSS1 *lctP1* and *lctP* mutant grew better than WT. There is precedent for this finding as well. *Haemophilus influenzae* is a facultative anaerobe and opportunistic pathogen that causes respiratory disease (Turk 1984). *H. influenzae ΔlctP* had a higher growth rate compared to WT in the mid-log phase & attained a higher OD during stationary phase in a L-lactate dependent manner (Lichtenegger et al. 2014) The authors speculated that this increased growth could be strain-specific but they determined that ArcAB, the two-component global regulon canonically associated with microbial lactate utilization genes, was not responsible for enhanced growth of the *ΔlctP* strain. *H. pylori* PMSS1 *ΔlctP* similarly outgrew WT independent from nutrient conditions or lactate supplementation (Fig. 3). Generally, we observed in a liquid growth curve that *H. pylori* PMSS1 *ΔLctP1* grew the best, followed by *ΔLctP,* then WT, with *ΔLctP2*

having the worst growth (Fig. 3). However, these in vitro growth phenotypes were starkly contrasting to the in vivo colonization phenotypes (Fig. 4).

In vivo, lactate uptake has been shown to promote growth and also help survive innate immune challenges. *H. influenzae* is reliant upon lactate utilization genes for in vivo colonization but loss of *lctP* does not alter complement resistance (Herbert et al. 2002)(Jiang et al. 2014) (Lichtenegger et al. 2014). *N. meningitidis* is dependent upon host endogenous L-lactate to colonize the nasopharynx (Harry Smith, Tang, and Exley 2007). Loss of *lctP* in *N. meningitidis* attenuated colonization and abrogated complement resistance in ex-vivo and murine in vivo models (Exley et al. 2005, 2007).

We observed in two separate 2-week murine in vivo infections significant mucosal and drastic glandular colonization defects in the *H. pylori* PMSS1 *lctP* mutants (Fig. 4A-B). Relative to PMSS1 WT mucosal colonization, PMSS1 *ΔlctP* had a significant colonization defect that was similar between the antrum and corpus while PMSS1 *ΔlctP2* only had a significant colonization defect in the antrum. In accordance with Sigal et al. 2015 and Keilberg et al. 2016, the *H. pylori* WT infection had greater glandular colonization in the antrum compared to the corpus. The glandular occupancy in the antrum and corpus of PMSS1 *ΔlctP2* infections was dramatically lower relative to WT (Fig. 4C-D). *ΔlctP2* glandular occupancy was noticeably lower in the antrum compared to the corpus, which is in contrast with the typical early-infection behavior of increased gastric colonization in the antrum relative to the corpus (Keilberg et al. 2016; Sigal et al. 2015). PMSS1 *ΔlctP* solely colonized 5% of the gastric glands in the corpus (Fig. 4C-D). Sigal et al. 2015 observed via 3D confocal microscopy that *H. pylori* gastric gland colonization rather than luminal

surface colonization precedes regional gastric pathogenesis. Additionally, Keilberg and Ottemann et al. 2016 reported that maintenance of chronic *H. pylori* infections is dependent upon exclusive glandular colonization in 10% of the gastric glands.

Curiously, PMSS1 *ΔlctP1* was unable to colonize the gastric mucous or glands despite displaying the best in vitro growth amongst the infection strains whereas PMSS1 *ΔlctP2* and *ΔlctP* successfully established an infection whilst respectively having the worst in vitro growth and lacking both permeases (Fig. 3&4). Iwatani et al. 2014 observed *H. pylori ΔlctP* to have moderately decreased consumption of L and D lactate relative to WT. This confirmed that *lctP1* and *lctP2* are responsible for some of the uptake of both lactate isomers, but the stereospecificity of each permease was left unknown, but also suggested there are other ways for lactate to enter the cells. This colonization defect of *ΔlctP1* strains could be attributed to a variety of factors. LctP1 could be primarily responsible for uptake of either one or both lactate enantiomers and the concurrent expression of *lctP2* could be preventing the activity of alternate permeases responsible for lactate uptake in the dual *lctP* deletion. *E. coli* glycolate permease can only uptake the D-lactate isomer and although *H. pylori* is not known to possess any alternative permeases responsible for lactate uptake, it's possible for an unknown permease to have its expression suppressed by the constitutively active lactate permeases (M. F. Núñez et al. 2001; María Felisa Núñez et al. 2002). Another possibility to consider is that LctP function is dependent upon downstream activity of LDH, as was observed in *H. influenzae* (Lichtenegger et al. 2014). Iwatani et al. 2014 reported that *H. pylori ΔL-LDH* and *ΔD-LDH* strains were unable to uptake their respective isomers, possibly indicating a protein / protein interaction between the lactate permease and

lactate dehydrogenase. Observing the colonization phenotype of a PMSS1 *ΔlctP1* complemented strain would be a strong indicator for the validity of our reported PMSS1 *ΔlctP1* colonization phenotype. Ultimately, the in vivo colonization defects observed in PMSS1 *LctP* mutants suggest that *H. pylori* is reliant upon nutritional virulence (Abu Kwaik and Bumann 2013).

8 Conclusions

The chemotactic and metabolic influence that L and D lactate imposed upon *H. pylori* could be critical for predicting bacterial localization within the gastric niche. In addition, elucidating the physiological and pathogenic roles of *H. pylori* lactate utilization genes can potentially lead to the identification of a novel *H. pylori* pathogenesis. Current studies are suggesting that further characterizing pathogenic manipulation of host metabolic activity would grant the ability to outline a detailed molecular pathway of the development of *H. pylori* mediated gastric cancer. This study presents an initial characterization of *H. pylori* lactate chemotaxis, as well as a foundation for determining the contributions that lactate utilization genes make towards *H. pylori* metabolism and gastric colonization.

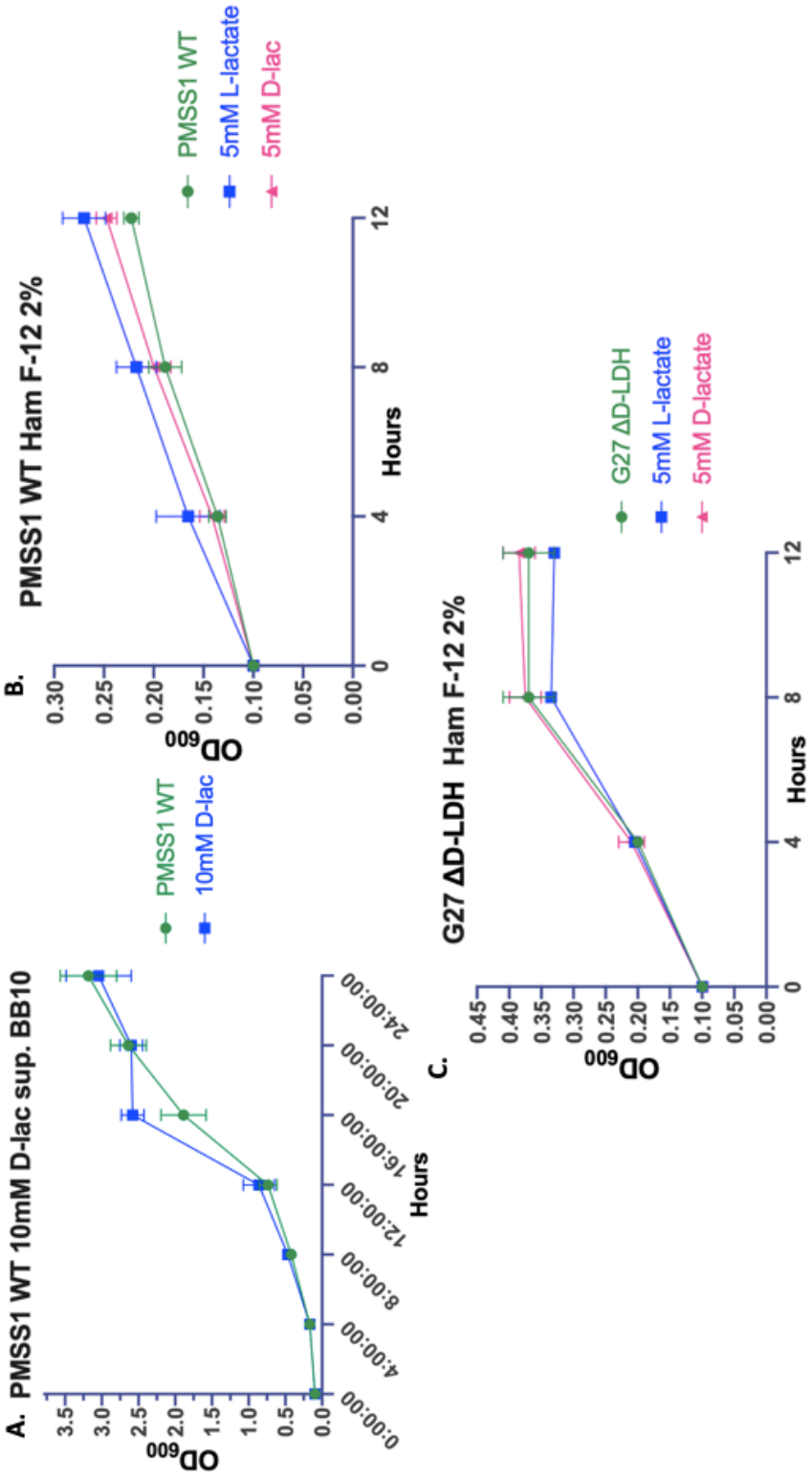
9 Recommendations for future study

1. Future studies should attempt measuring the lactate concentration gradient within the murine gastric region. This would provide immense insight towards the physiological environment that host gastric cells and *H. pylori* alike are subject to within the host, overall providing much needed context towards phenotypes observed within in vitro chemotaxis / growth curve assays and in vivo infections.

2. The metabolic function of *H. pylori* D-LDH must conclusively be determined in order to have a general understanding of the basic function of *H. pylori* lactate utilization genes. Additionally, the stereospecificity of the individual *H. pylori* lactate permeases should be characterized
3. The PMSS1 *ΔlctP1* mutant should have its complimented strain undergo growth analysis in vitro and in vivo murine infections to determine if the lack of gastric colonization and growth effects observed in this study should undergo further consideration geared towards elucidating a molecular mechanism.

10 Appendix

Supplemental Figure 1. Liquid growth curve of lactate supplementation in PMSS1 WT grown in BB10 (a) and Ham F-12 2% (b), as well as G27 *ΔD-LDH* in Ham F-12 2%.


A. PMSS1 WT 10mM D-lac sup. BB10
PMSS1 WT
10mM D-lac
OD600
Hours
B. PMSS1 WT Ham F-12 2%
PMSS1 WT
5mM L-lactate
5mM D-lac
OD600
Hours
C. G27 ΔD-LDH Ham F-12 2%
G27 ΔD-LDH
5mM L-lactate
5mM D-lactate
OD600
Hours

11 Citations

Abu Kwaik, Yousef, and Dirk Bumann. 2013. “Microbial Quest for Food *in Vivo* : ‘Nutritional Virulence’ as an Emerging Paradigm.” *Cellular Microbiology* 15(6): 882–90. http://doi.wiley.com/10.1111/cmi.12138 (March 16, 2021).

Akeel, Mohammed et al. 2019. “Helicobacter Pylori VacA, CagA and IceA Genotypes in Dyspeptic Patients from Southwestern Region, Saudi Arabia: Distribution and Association with Clinical Outcomes and Histopathological Changes.” *BMC Gastroenterology* 19(1).

Akram, M. 2013. “Mini-Review on Glycolysis and Cancer.” *Journal of Cancer Education* 28(3): 454–57.

Aldunate, Muriel et al. 2015. “Antimicrobial and Immune Modulatory Effects of Lactic Acid and Short Chain Fatty Acids Produced by Vaginal Microbiota Associated with Eubiosis and Bacterial Vaginosis.” *Frontiers in Physiology* 6(JUN).

Andermann, Tessa M., Yu Ting Chen, and Karen M. Ottemann. 2002. “Two Predicted Chemoreceptors of Helicobacter Pylori Promote Stomach Infection.” *Infection and Immunity* 70(10): 5877–81.

Baker, Ann Marie et al. 2015. “Characterization of LGR5 Stem Cells in Colorectal Adenomas and Carcinomas.” *Scientific Reports* 5(1): 1–8. www.nature.com/scientificreports (March 15, 2021).

Barker, Nick et al. 2010. “Lgr5+ve Stem Cells Drive Self-Renewal in the

Stomach and Build Long-Lived Gastric Units In Vitro." *Cell Stem Cell* 6(1): 25–36.

Bianchetti, Davide G.A.M. et al. 2018. "D-Lactic Acidosis in Humans: Systematic Literature Review." *Pediatric Nephrology* 33(4): 673–81.

Bury-Moné, Stephanie et al. 2006. "Is Helicobacter Pylori a True Microaerophile?" *Helicobacter* 11(4): 296–303.

Censini, Stefano et al. 1996. "Cag, a Pathogenicity Island of Helicobacter Pylori, Encodes Type I-Specific and Disease-Associated Virulence Factors." *Proceedings of the National Academy of Sciences of the United States of America* 93(25): 14648–53.

Chang, Huai-Tzu et al. 1995. "Kinetics of Substrate Oxidation by Whole Cells and Cell Membranes of *Helicobacter Pylori*." *FEMS Microbiology Letters* 129(1): 34–38. https://academic.oup.com/femsle/article-lookup/doi/10.1111/j.1574-6968.1995.tb07553.x (September 20, 2019).

Collins, Kieran D. et al. 2016. "The Helicobacter Pylori CZB Cytoplasmic Chemoreceptor TlpD Forms an Autonomous Polar Chemotaxis Signaling Complex That Mediates a Tactic Response to Oxidative Stress." *Journal of Bacteriology* 198(11): 1563–75.

Cormedi, Marina Candido Visontai et al. 2018. "Survival and Prognosis of Young Adults with Gastric Cancer." *Clinics* 73.

Cristescu, Melania E., David J. Innes, Jonathon H. Stillman, and Teresa J. Crease. 2008. "D- and L-Lactate Dehydrogenases during Invertebrate

Evolution.” *BMC Evolutionary Biology* 8(1).

Dong, J M et al. 1993. 175 JOURNAL OF BACrERIOLOGY *Three Overlapping Ict Genes Involved in L-Lactate Utilization by Escherichia Coli Downloaded From*. http://jb.asm.org/ (May 21, 2020).

Edelstein, Arthur D et al. 2014. “Advanced Methods of Microscope Control Using MManager Software.” *Journal of Biological Methods* | 1(2): 10. www.jbmethods.org (March 8, 2021).

El-Omar, Emad M., Wong□Ho H. Chow, and Charles S. Rabkin. 2001. “Gastric Cancer and H. Pylori: Host Genetics Open the Way.” *Gastroenterology* 121(4): 1002–4.

Ewaschuk, Julia B., Jonathan M. Naylor, and Gordon A. Zello. 2005. “D-Lactate in Human and Ruminant Metabolism.” *The Journal of Nutrition* 135(7): 1619–25.

Exley, Rachel M. et al. 2005. “Neisseria Meningitidis Lactate Permease Is Required for Nasopharyngeal Colonization.” *Infection and Immunity* 73(9): 5762–66. http://iai.asm.org/ (October 2, 2020).

Exley, Rachel M et al. 2007. “Lactate Acquisition Promotes Successful Colonization of the Murine Genital Tract by Neisseria Gonorrhoeae.” *INFECTION AND IMMUNITY* 75(3): 1318–24. www.genome.ou.edu/gono.html (October 2, 2020).

Fantin, Valeria R., Julie St-Pierre, and Philip Leder. 2006. “Attenuation of LDH-A Expression Uncovers a Link between Glycolysis, Mitochondrial

Physiology, and Tumor Maintenance." *Cancer Cell* 9(6): 425–34.

Flick, Matthew J., and Stephen F. Konieczny. 2002. "Identification of Putative Mammalian D-Lactate Dehydrogenase Enzymes." *Biochemical and Biophysical Research Communications* 295(4): 910–16.

Futai, Masamitsu, and J Biol. 1972. 49 J. Biol. Chem. 245,6649. J. Haematol *Membrane D-Lactate Dehydrogenase from Purification and Propertiest*. Academic Press. https://pubs.acs.org/sharingguidelines (March 16, 2021).

Heinová, Dagmar, Zuzana Kostecká, and Eva Petrovová. 2018. "Lactate Dehydrogenase Isoenzyme Electrophoretic Pattern in Serum and Tissues of Mammalian and Bird Origin." In *Electrophoresis - Life Sciences Practical Applications*, InTech.

Herbert, M. A. et al. 2002. "Signature Tagged Mutagenesis of Haemophilus Influenzae Identifies Genes Required for in Vivo Survival." *Microbial Pathogenesis* 33(5): 211–23.

Hohenberger, Peter, and Stephan Gretschel. 2003. "Gastic Cancer." *The Lancet* 362(9380): 305–15. https://linkinghub.elsevier.com/retrieve/pii/S014067360313975X (September 5, 2019).

Hosoda, F. et al. 2015. "Integrated Genomic and Functional Analyses Reveal Glyoxalase I as a Novel Metabolic Oncogene in Human Gastric Cancer." *Oncogene* 34(9): 1196–1206.

Huang, Julie Y., Emily Goers Sweeney, Karen Guillemin, and Manuel R. Amieva. 2017. “Multiple Acid Sensors Control Helicobacter Pylori Colonization of the Stomach.” *PLoS Pathogens* 13(1).

Humphries, Adam et al. 2013. “Lineage Tracing Reveals Multipotent Stem Cells Maintain Human Adenomas and the Pattern of Clonal Expansion in Tumor Evolution.” *Proceedings of the National Academy of Sciences of the United States of America* 110(27): E2490–99. www.pnas.org/cgi/doi/10.1073/pnas.1220353110 (March 15, 2021).

Ilina, Elena N. et al. 2010. “Application of Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry for the Study of Helicobacter Pylori.” *Rapid Communications in Mass Spectrometry*.

Iwatani, Shun et al. 2014. “Identification of the Genes That Contribute to Lactate Utilization in Helicobacter Pylori.” *PLoS ONE* 9(7).

Jäger, Doris et al. 2000. “Two-Dimensional Electrophoretic and Immunoblot Analysis of Cell Surface Proteins of Spiral-Shaped Coccoid Forms of Helicobacter Pylori.” *Electrophoresis* 21(13): 2670–77.

Jiang, Tianyi et al. 2017. “A Bacterial Multidomain NAD-Independent d-Lactate Dehydrogenase Utilizes Flavin Adenine Dinucleotide and Fe-S Clusters as Cofactors and Quinone as an Electron Acceptor for d-Lactate Oxidization.” *Journal of bacteriology* 199(22). http://www.ncbi.nlm.nih.gov/pubmed/28847921 (September 26, 2019).

Jiang, Tianyi, Chao Gao, Cuiqing Ma, and Ping Xu. 2014. “Microbial Lactate

Utilization: Enzymes, Pathogenesis, and Regulation." *Trends in Microbiology* 22(10): 589–99.

Karlsson, Anneli et al. 2012. "Association between CagA and VacA Genotypes and Pathogenesis in a Helicobacter Pylori Infected Population from South-Eastern Sweden." *BMC Microbiology* 12(1): 129. http://bmcmicrobiol.biomedcentral.com/articles/10.1186/1471-2180-12-129 (September 5, 2019).

Kato, Osamu et al. 2010. "Quinone-Dependent D-Lactate Dehydrogenase Dld (Cg1027) Is Essential for Growth of Corynebacterium Glutamicum on D-Lactate." *BMC microbiology* 10(1): 321. http://bmcmicrobiol.biomedcentral.com/articles/10.1186/1471-2180-10-321 (March 16, 2021).

Keenan, Jacqueline I., Nina Salm, Mark B. Hampton, and Alison J. Wallace. 2010. "Individual and Combined Effects of Foods on Helicobacter Pylori Growth." *Phytotherapy Research* 24(8): 1229–33.

Keilberg, Daniela et al. 2016. "Spatial and Temporal Shifts in Bacterial Biogeography and Gland Occupation during the Development of a Chronic Infection." *mBio* 7(5).

Keilberg, Daniela, and Karen M. Ottemann. 2016. "How Helicobacter Pylori Senses, Targets and Interacts with the Gastric Epithelium." *Environmental Microbiology* 18(3): 791–806.

Kelly, David J., Nicky J. Hughes, and Robert K. Poole. 2014. "Microaerobic

Physiology: Aerobic Respiration, Anaerobic Respiration, and Carbon Dioxide Metabolism." In *Helicobacter Pylori*, Washington, DC, USA: ASM Press, 111–24. http://doi.wiley.com/10.1128/9781555818005.ch10 (February 18, 2021).

Kim, Hee Sung, Hee Eun Lee, Han Kwang Yang, and Woo Ho Kim. 2014. "High Lactate Dehydrogenase 5 Expression Correlates with High Tumoral and Stromal Vascular Endothelial Growth Factor Expression in Gastric Cancer." *Pathobiology* 81(2): 78–85.

Kozar, Sarah et al. 2013. "Continuous Clonal Labeling Reveals Small Numbers of Functional Stem Cells in Intestinal Crypts and Adenomas." *Cell stem cell* 13(5): 626–33.

Lee, Do Yeon et al. 2017. "Regulation of SIRT3 Signal Related Metabolic Reprogramming in Gastric Cancer by Helicobacter Pylori Oncoprotein CagA." *Oncotarget* 8(45): 78365–78.

Lee, Woon Ching, Khean Lee Goh, Mun Fai Loke, and Jamuna Vadivelu. 2017. "Elucidation of the Metabolic Network of Helicobacter Pylori J99 and Malaysian Clinical Strains by Phenotype Microarray." *Helicobacter* 22(1).

Lee, Yong-Soo et al. 2018. "Microbiota-Derived Lactate Accelerates Intestinal Stem-Cell-Mediated Epithelial Development." *Cell host & microbe* 24(6): 833-846.e6. http://www.ncbi.nlm.nih.gov/pubmed/30543778 (September 21, 2019).

Lichtenegger, Sabine et al. 2014. “Characterization of Lactate Utilization and Its Implication on the Physiology of Haemophilus Influenzae.” *International Journal of Medical Microbiology* 304(3–4): 490–98.

Liu, Yuanda et al. 2019. “Metabolic Reprogramming Results in Abnormal Glycolysis in Gastric Cancer: A Review.” *OncoTargets and Therapy* 12: 1195–1204.

Lo, H, CM Tang, RM Exley - The Lancet infectious diseases, and undefined 2009. “Mechanisms of Avoidance of Host Immunity by Neisseria Meningitidis and Its Effect on Vaccine Development.” *Elsevier*. https://www.sciencedirect.com/science/article/pii/S147330990970132X (February 17, 2021).

Louis, Petra, Georgina L. Hold, and Harry J. Flint. 2014. “The Gut Microbiota, Bacterial Metabolites and Colorectal Cancer.” *Nature Reviews Microbiology* 12(10): 661–72.

Luo, Bin et al. 2016. “ATP-Dependent Lon Protease Contributes to Helicobacter Pylori-Induced Gastric Carcinogenesis.” *Neoplasia (United States)* 18(4): 242–52.

Machuca, Mayra A. et al. 2017. “Helicobacter Pylori Chemoreceptor TlpC Mediates Chemotaxis to Lactate.” *Scientific Reports* 7(1).

Mahe, Maxime M. et al. 2013. “Establishment of Gastrointestinal Epithelial Organoids.” *Current Protocols in Mouse Biology* 3(4): 217–40. http://doi.wiley.com/10.1002/9780470942390.mo130179 (March 8,

2021).

Mavric, Elvira, Silvia Wittmann, Gerold Barth, and Thomas Henle. 2008. "Identification and Quantification of Methylglyoxal as the Dominant Antibacterial Constituent of Manuka (Leptospermum Scoparium) Honeys from New Zealand." *Molecular nutrition & food research* 52(4): 483–89. http://www.ncbi.nlm.nih.gov/pubmed/18210383 (September 20, 2019).

Mendz, G L, S L Hazell, and L van Gorkom. 1994. "Pyruvate Metabolism in Helicobacter Pylori." *Archives of microbiology* 162(3): 187–92. http://www.ncbi.nlm.nih.gov/pubmed/7979873 (September 6, 2019).

Monroe, Glen R. et al. 2019. "Identification of Human D Lactate Dehydrogenase Deficiency." *Nature Communications* 10(1).

Núñez, M. F. et al. 2001. "The Gene YghK Linked to the Glc Operon of Escherichia Coli Encodes a Permease for Glycolate That Is Structurally and Functionally Similar to L-Lactate Permease." *Microbiology* 147(4): 1069–77. https://www.microbiologyresearch.org/content/journal/micro/10.1099/00221287-147-4-1069 (February 19, 2021).

Núñez, María Felisa et al. 2002. "Transport of L-Lactate, D-Lactate, and Glycolate by the LldP and GlcA Membrane Carriers of Escherichia Coli." *Biochemical and Biophysical Research Communications* 290(2): 824–29.

Ottemann, Karen M., and Andrew C. Lowenthal. 2002. "Helicobacter Pylori Uses Motility for Initial Colonization and to Attain Robust Infection."

Infection and Immunity 70(4): 1984–90. https://pubmed.ncbi.nlm.nih.gov/11895962/ (March 20, 2021).

Parrish, Carol Rees. 2015. Series Editor PRACTICAL GASTROENTEROLOGY *NUTRITION ISSUES IN GASTROENTEROLOGY, SERIES #145 D-Lactic Acidosis: More Prevalent Than We Think?* www.mayomedicallaboratories.com (September 6, 2019).

Parsons, Bryony N. et al. 2017. "Comparison of the Human Gastric Microbiota in Hypochlorhydric States Arising as a Result of Helicobacter Pylori-Induced Atrophic Gastritis, Autoimmune Atrophic Gastritis and Proton Pump Inhibitor Use." *PLoS Pathogens* 13(11).

Phesse, Toby J., and Owen J. Sansom. 2017. "Lgr5 Joins the Club of Gastric Stem Cell Markers in the Corpus." *Nature Cell Biology* 19(7): 752–54. https://www.nature.com/articles/ncb3567 (March 16, 2021).

Pinchuk, Grigoriy E. et al. 2011. "Pyruvate and Lactate Metabolism by Shewanella Oneidensis MR-1 under Fermentation, Oxygen Limitation, and Fumarate Respiration Conditions." *Applied and Environmental Microbiology* 77(23): 8234–40. http://aem.asm.org/.http://aem.asm.org/ (October 1, 2020).

Pinchuk, Grigory E. et al. 2009. "Genomic Reconstruction of Shewanella Oneidensis MR-1 Metabolism Reveals a Previously Uncharacterized Machinery for Lactate Utilization." *Proceedings of the National Academy*

of Sciences of the United States of America 106(8): 2874–79.

Pitson, Stuart M., George L. Mendz, Sujatha Srinivasan, and Stuart L. Hazell. 1999. “The Tricarboxylic Acid Cycle of Helicobacter Pylori.” *European Journal of Biochemistry* 260(1): 258–67.

Rajeev, Lara et al. 2019. “LurR Is a Regulator of the Central Lactate Oxidation Pathway in Sulfate-Reducing Desulfovibrio Species” ed. Z. Tom Wen. *PLOS ONE* 14(4): e0214960. https://dx.plos.org/10.1371/journal.pone.0214960 (February 18, 2021).

Rodríguez-Colman, Maria J. et al. 2017. “Interplay between Metabolic Identities in the Intestinal Crypt Supports Stem Cell Function.” *Nature* 543(7645): 424–27. https://www.nature.com/articles/nature21673 (March 15, 2021).

Rolig, Annah S., James Shanks, J. Elliot Carter, and Karen M. Ottemann. 2012. “Helicobacter Pylori Requires TlpD-Driven Chemotaxis to Proliferate in the Antrum.” *Infection and Immunity* 80(10): 3713–20.

Schepers, Arnout G. et al. 2012. “Lineage Tracing Reveals Lgr5+ Stem Cell Activity in Mouse Intestinal Adenomas.” *Science* 337(6095): 730–35. http://science.sciencemag.org/ (March 15, 2021).

Schilling, Christophe H. et al. 2002. “Genome-Scale Metabolic Model of Helicobacter Pylori 26695.” *Journal of Bacteriology* 184(16): 4582–93.

Schoen, Christoph, Laura Kischkies, Johannes Elias, and Biju Joseph Ampattu. 2014. “Metabolism and Virulence in Neisseria Meningitidis.”

Frontiers in Cellular and Infection Microbiology 4(AUG).

Schreiber, S, M Konradt, … C Groll - Proceedings of the, and undefined 2004. “The Spatial Orientation of Helicobacter Pylori in the Gastric Mucus.” *National Acad Sciences*.

Schreiber, Sören et al. 2004. “The Spatial Orientation of Helicobacter Pylori in the Gastric Mucus.” *Proceedings of the National Academy of Sciences of the United States of America* 101(14): 5024–29. www.pnas.orgcgidoi10.1073pnas.0308386101 (March 14, 2021).

Schulz, Christian et al. 2016. “The Active Bacterial Assemblages of the Upper Gi Tract in Individuals with and without Helicobacter Infection.” *Gut* 67(2): 216–25. https://gut.bmj.com/content/67/2/216 (February 17, 2021).

Sigal, Michael et al. 2015. “Helicobacter Pylori Activates and Expands Lgr5+ Stem Cells through Direct Colonization of the Gastric Glands.” *Gastroenterology* 148(7): 1392-1404.e21.

Sigurlásdóttir, Sara et al. 2019. “Deletion of D-Lactate Dehydrogenase A in Neisseria Meningitidis Promotes Biofilm Formation through Increased Autolysis and Extracellular DNA Release.” *Frontiers in Microbiology* 10(MAR).

Silva, Marta Sousa et al. 2013. “The Glyoxalase Pathway: The First Hundred Years. .. and Beyond.” *Biochem. J* 453: 1–15.

Smith, Brian R., and Bruce E. Stabile. 2009. “Extreme Aggressiveness and Lethality of Gastric Adenocarcinoma in the Very Young.” *Archives of*

Surgery 144(6): 506–10.

Smith, H., E. A. Yates, J. A. Cole, and N. J. Parsons. 2001. “Lactate Stimulation of Gonococcal Metabolism in Media Containing Glucose: Mechanism, Impact on Pathogenicity, and Wider Implications for Other Pathogens.” *Infection and Immunity* 69(11): 6565–72. http://iai.asm.org/ (February 17, 2021).

Smith, Harry, Christoph M Tang, and Rachel M Exley. 2007. “Effect of Host Lactate on Gonococci and Meningococci: New Concepts on the Role of Metabolites in Pathogenicity Downloaded From.” *INFECTION AND IMMUNITY* 75(9): 4190–98. http://iai.asm.org/ (February 18, 2021).

Stein, Markus, Rino Rappuoli, and Antonello Covacci. 2014. “The Cag Pathogenicity Island.” In *Helicobacter Pylori*, American Society of Microbiology, 345–53.

Sugiyama, Naoyuki et al. 2019. “Comparative Proteomics of Helicobacter Pylori Strains Reveals Geographical Features Rather than Genomic Variations.” *Genes to Cells* 24(2): 139–50.

Sun, Shiren, Heng Li, Jianghua Chen, and Qi Qian. 2017. “Lactic Acid: No Longer an Inert and End-Product of Glycolysis.” *Physiology* 32(6): 453–63.

Takahashi, Tetsufumi et al. 2007. “L-Lactic Acid Secreted from Gastric Mucosal Cells Enhances Growth of Helicobacter Pylori.” *Helicobacter* 12(5): 532–40.

Takaji, S. et al. 1997. “Growth Inhibition of Helicobacter Pylori by a Polyamine Synthesis Inhibitor, Methylglyoxal Bis(Cyclopentylamidinohydrazone).” *Letters in Applied Microbiology* 25(3): 177–80.

Tang, Yinjie et al. 2007. “Pathway Confirmation and Flux Analysis of Central Metabolic Pathways in Desulfovibrio Vulgaris Hildenborough Using Gas Chromatography-Mass Spectrometry and Fourier Transform-Ion Cyclotron Resonance Mass Spectrometry.” *Journal of Bacteriology* 189(3): 940–49. http://jb.asm.org/ (February 18, 2021).

Thomas, Marie T. et al. 2011. “Two Respiratory Enzyme Systems in Campylobacter Jejuni NCTC 11168 Contribute to Growth on L-Lactate.” *Environmental Microbiology* 13(1): 48–61.

Thornalley, Paul J. 1993. “The Glyoxalase System in Health and Disease.” *Molecular Aspects of Medicine* 14(4): 287–371.

Thornalley, Paul J. 1990. 269 Biochem. J *The Glyoxalase System: New Developments towards Functional Characterization of a Metabolic Pathway Fundamental to Biological Life**.

Tomb, Jean F. et al. 1997. “The Complete Genome Sequence of the Gastric Pathogen Helicobacter Pylori.” *Nature* 388(6642): 539–47.

Turk, D. C. 1984. “The Pathogenicity of Haemophilus Influenzae.” *Journal of Medical Microbiology* 18(1): 1–16. https://www.microbiologyresearch.org/content/journal/jmm/10.1099/00222615-18-1-1 (March 16, 2021).

Uehara, Takeshi et al. 2013. “H. Pylori Infection Is Associated with DNA Damage of Lgr5-Positive Epithelial Stem Cells in the Stomach of Patients with Gastric Cancer.” *Digestive Diseases and Sciences* 58(1): 140–49. https://link.springer.com/article/10.1007/s10620-012-2360-8 (March 16, 2021).

Venkateswaran, Kasthuri et al. 1999. “Polyphasic Taxonomy of the Genus Shewanella and Description of Shewanella Oneidensis Sp. Nov.” *International Journal of Systematic Bacteriology* 49(2): 705–24. https://www.microbiologyresearch.org/content/journal/ijsem/10.1099/00207713-49-2-705 (March 15, 2021).

Vinasco, Karla, Hazel M. Mitchell, Nadeem O. Kaakoush, and Natalia Castaño-Rodríguez. 2019. “Microbial Carcinogenesis: Lactic Acid Bacteria in Gastric Cancer.” *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer* 1872(2): 188309.

Vita, Nicolas et al. 2015. “The Primary Pathway for Lactate Oxidation in Desulfovibrio Vulgaris.” *Frontiers in Microbiology* 6(JUN): 606. http://journal.frontiersin.org/Article/10.3389/fmicb.2015.00606/abstract (February 18, 2021).

Wadhams, George H., and Judith P. Armitage. 2004. “Making Sense of It All: Bacterial Chemotaxis.” *Nature Reviews Molecular Cell Biology* 5(12): 1024–37.

Walenta, Stefan, and Wolfgang F Mueller-Klieser. 2004. “Lactate: Mirror and

Motor of Tumor Malignancy." *Seminars in radiation oncology* 14(3): 267–74. http://www.ncbi.nlm.nih.gov/pubmed/15254870 (September 6, 2019).

Wang, Fei, Wenbo Meng, Bingyuan Wang, and Liang Qiao. 2014. "Helicobacter Pylori-Induced Gastric Inflammation and Gastric Cancer." *Cancer Letters* 345(2): 196–202.

Wang, Xiangfei et al. 2018. "LGR5 Regulates Gastric Adenocarcinoma Cell Proliferation and Invasion via Activating Wnt Signaling Pathway." *Oncogenesis* 7(8): 57. https://www.nature.com/articles/s41389-018-0071-5 (March 16, 2021).

Wang, Yufeng et al. 2012. "Glyoxalase I (GLO1) Is up-Regulated in Pancreatic Cancerous Tissues Compared with Related Non-Cancerous Tissues." *Anticancer research* 32(8): 3219–22. http://www.ncbi.nlm.nih.gov/pubmed/22843895 (September 20, 2019).

Xi, Hong Qing et al. 2014. "Increased Expression of Lgr5 Is Associated with Chemotherapy Resistance in Human Gastric Cancer." *Oncology Reports* 32(1): 181–88. http://www.spandidos-publications.com/10.3892/or.2014.3207/abstract (March 16, 2021).

Xiang, Zhaoying et al. 1995. 63 INFECTION AND IMMUNITY *Analysis of Expression of CagA and VacA Virulence Factors in 43 Strains of Helicobacter Pylori Reveals That Clinical Isolates Can Be Divided into Two Major Types and That CagA Is Not Necessary for Expression of the Vacuolating Cytotoxin*. http://iai.asm.org/ (September 5, 2019).

Yeon BAEK, Hye et al. 2004. 379 Biochem. J *Oxidative-Stress-Related Proteome Changes in Helicobacter Pylori-Infected Human Gastric Mucosa*. http://www.matrixscience.com (September 21, 2019).