Investigations into the Effects of Lactoferrin on Microbial Ecology, using *Helicobacter pylori* as a Model Organism

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Table of Contents

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Abstract

Lactoferrin (Lf) is an iron binding protein produced in mammals. It has antimicrobial and immunomodulatory properties. Some bacteria that regularly colonize mammalian hosts have adapted to living in high Lf environments. *Helicobacter pylori*, which inhabits the human gut, was chosen as a model organism to investigate how bacteria may adapt to Lf.

H. pylori was able to use iron from fully saturated human Lf (hLf) in various low iron media, achieving growth levels similar to the iron-replete control. Partially saturated hLf decreased growth, yet both partially saturated bovine Lf (bLf) and hLf were able to increase internalization of bacteria into mammalian tissue culture cells. A substantially larger increase in internalization was seen when bacteria were supplemented with hLf in low iron conditions, possibly mediated by iron-regulated cellular receptors or bacterial lactoferrin binding proteins.

In eukaryotes, Lf is known to bind and facilitate internalization of DNA into cells and sometimes the nucleus, and upregulate gene expression. Here, one hundred bacterial genomes were surveyed for known Lf binding sites as an indication that Lf had similar functions using bacterial DNA. While the frequency and location of Lf binding sites suggest they occur at random, their presence in all genomes suggests that Lf may be able to act as a vector for bacterial DNA, and facilitate the movement of genes between species.

Lf is being widely considered for commercial and therapeutic uses, with significant interest in producing it in genetically modified organisms (GMO). Widespread production and use of Lf could increase the number of bacteria that are adapted to it. How Lf interacts with bacteria adapted to it, and the ability of it to act as a DNA vector, may have relevance for GMO risk assessment.

List of Figures

List of Tables

Abbreviations

AGS	Gastric adenocarcinoma cells
BB	Brucella broth
bLf	Bovine lactoferrin
CagA	Cytotoxin associated gene product A
DE	Desferoxamine mesylate
DNA	Deoxyribose nucleic acid
FCS	Fetal bovine serum
hLf	Human lactoferrin
IL	Iron-limited
IROMPs	Iron-regulated outer membrane proteins
IR	Iron-replete
kDa	Kilo-dalton
LPS	Lipopolysaccharides
Lf	Lactoferrin
Lfcin	Lactoferricin
PBS	Phosphate buffered saline
rhLf	Recombinant human lactoferrin
SDS	Sodium dodecyl sulphate
Tf	Transferrin
VacA	Vacuolating cytotoxin A

Chapter 1
Introduction

Lactoferrin (Lf) is a multifunctional iron-binding protein produced by mammals and found in glandular secretions such as milk, on mucosal surfaces, and in neutrophils (Ling and Schryvers 2006). In eukaryotic cells, Lf is proposed to regulate aspects of the cell cycle, assist in iron-uptake in the intestine, modulate the immune response to infection, and, in some cases, directly activate gene expression as a transcription factor in the nucleus (He and Furmanski 1995). However, Lf also comes into contact with pathogenic and commensal bacteria on mucosal surfaces and at sites of infection, where it can interact with these bacteria in a number of ways. It can inhibit bacterial growth by sequestering free iron in the extracellular environment (Ward *et al.* 2005). Lactoferrin can also exhibit protease activity against bacterial virulence factors, abrogate invasion in a number of species and increase bacterial membrane permeability (which can be bactericidal) (Valenti and Antonini 2005).

Thus, with Lf's many effects on bacteria and the immune system, there is significant interest in using it as a therapeutic agent. Biopharming (engineering plants and animals to produce pharmaceuticals) has been considered as a potential source of mass-produced, cost-effective Lf. However, the use of genetically engineered organisms to produce Lf in large quantities within the natural environment would expose a different range of bacteria to Lf and the molecules to which it binds. Therefore our ability to assess the risks of undertakings such as biopharming first relies on a solid understanding of Lf's interactions with prokaryotes.

1.1 The Ability of Lactoferrin to Affect Microbial Growth

1.1.1 The Structure and Iron Binding Properties of Lactoferrin

Lactoferrin was first isolated from milk, and named for both its source (lacto) and its ability to bind iron (ferrin). It is constitutively expressed at the highest levels in colostrum and milk, with lower levels expressed in tears, nasal fluids, saliva, and secretions from pancreatic, gastrointestinal, and reproductive tissues (Masson *et al.* 1966). Lactoferrin is also expressed in developing neutrophils and stored in secondary granules at a concentration of 3 g per 10^6 cells (Masson *et al.* 1969). Transcription of Lf is regulated by different environmental cues in differing tissues. These cues include growth factors, developmental cues, retinoic acid, and, in the reproductive organs, estrogen (Teng 2006).

Lf is an 80 kDa glycoprotein, comprised of a single polypeptide chain that folds into homologous N and C terminal lobes (Metz-Boutigue *et al.*1984). It is highly conserved across mammals, with an amino acid sequence that puts it in the larger family of iron-binding proteins, which includes transferrin (Tf) that is found in serum (Metze-Boutigue *et al.* 1984, Baker and Baker 2005). Lactoferrin is polycationic, with a particularly cationic region in its N-terminal lobe that is responsible for its ability to bind numerous substrates, including DNA, bacterial lipopolysaccharide (LPS), and heparin (Valenti and Antonini 2005).

This highly cationic region, which is one of the features that distinguish Lf from other members of the iron-binding protein family, can be released through pepsin hydrolysis. This results in the formation of an Lf-derived peptide termed lactoferricin (Lfcin) that retains many of the activities of the intact protein and in some cases displays an increased potency (Bellamy *et al.* 1992, Gifford *et al.* 2005). In solution, Lfcin adopts a structure different from its conformation on the intact protein. Its

amphipathic structure is similar to that of other peptides that display antimicrobial activity (Ward *et al.* 2005) and may account for the considerable antimicrobial properties that Lfcin displays against Gram-negative and Gram-positive bacteria, yeast, fungi, viruses, and protozoa (Bellamy *et al.* 1992, Ward *et al.* 2005). .

The two homologous lobes of Lf have conserved iron-binding sites that can reversibly bind ferric iron at a very high affinity ($K \sim 10^{22}$ M) (Mazurier and Spik 1980, Baker and Baker 2005). The binding of iron is accompanied by a conformational change in the Lf protein to a more closed structure, with the two domains of each lobe enclosing an iron ion and effectively sequestering it away from the external environment (Baker and Baker 2005). Lactoferrin is capable of retaining bound iron in acidic conditions as low as pH 3-4. In contrast, transferrin releases iron at about pH 5-6 (Mazurier and Spik 1980).

1.1.2 Inhibition of Bacterial Growth by Lactoferrin

Lactoferrin has a number of antimicrobial properties, well documented both *in vitro* and *in vivo*, many of which are related to its ability to bind iron (Singh *et al.* 2002, Ward *et al.* 2005). Iron is an essential element for almost all living organisms, acting as a cofactor for the activity of numerous enzymes, and as a catalyst in electron transport. One of the difficulties many organisms face in acquiring iron is that the soluble form of iron, ferrous iron (Fe(II)), becomes oxidized to the extremely insoluble ferric iron (Fe(III)) in the presence of oxygen. Ferric iron has a solubility of just 10^{-17} M at physiological pH, whereas bacteria generally require iron at around 10^{-7} to 10^{-5} M to achieve maximal growth (Andrews *et al.* 2003).

The situation can be particularly dire for bacteria that colonize mammalian hosts, where most remaining free iron in serum and on mucosal surfaces is chelated by the host iron-binding proteins Tf and Lf,

respectively (Masson *et al.* 1966), making iron in the body a particularly precious resource for bacteria (Raymond *et al.* 2003, Miethke and Marahiel 2007). Indeed, bacteriostasis (inhibition of bacterial growth) was the first antimicrobial property described for Lf (Ward *et al.* 2005). This is because Lf exists primarily in the apo, non-iron bound state in the body and is able to readily chelate any free iron on mucosal surfaces. This property of Lf may also be part of the normal host resistance to biofilm formation, which can require high concentrations of iron (Reid *et al.* 2008). Iron sequestering by Lf on mucosal surfaces can inhibit formation of *Pseudomonas aeruginosa* biofilms, even in concentrations too low to affect growth rates (Singh *et al.* 2002).

However, not all of Lf's anti-microbial activity is due to binding iron. Lactoferrin displays bactericidal activity that is irreversible with the addition of iron, and thus distinct from its iron-sequestering, bacteriostatic properties (Arnold *et al.* 1982). The mechanism appears to be similar for both Gram-positive and Gram-negative bacteria, and involves the disruption of bacterial membranes. In Gram-negative bacteria, the N-terminal lobe of intact Lf is capable of inducing the release of lipopolysaccharides (LPS) from the outer membrane. This is done either directly, through binding the lipid A part of LPS, or indirectly, by chelating extracellular Ca^{2+} (Ellison *et al.* 1988, Appelmelk *et al.* 1994, Valenti and Antonini 2005). The release of LPS increases the permeability of the membrane, and the susceptibility of the bacteria to osmotic shock, lysozyme, and antibacterial molecules (Ellison *et al.*1988, Ward *et al.* 2005).

In Gram-positive bacteria, Lf probably acts by binding the lipid matrix of the cell surface via electrostatic interactions. The non-polar membrane interior becomes perturbed, leading to a similar increase in membrane permeability (Valenti and Antonini 2005). As the N-terminal region of Lf appears to be central for its effect on bacterial membranes, it is

not surprising that the N-terminal peptide Lfcin has a similar, if greatly amplified, effect (Valenti and Antonini 2005). In fact, along with increasing membrane permeability, Lfcin may be able to actually cross both the outer lipid layer and the cytoplasmic membrane of bacteria, possibly then acting on intracellular targets (Gifford *et al.* 2005).

1.1.3 Bacterial use of Lactoferrin as an Iron Source

To survive in the low-iron host environment, pathogens engage a wide array of methods for acquiring iron. Among these, some bacteria can scavenge iron from host proteins such as Tf, heme, and Lf. A number of bacteria reportedly use Lf as an iron source and human Lf-specific, membrane-bound, Lf binding proteins (LBPs) have been reported in *Neisseria meningitidis, N. gonorrhoeae, Bordetella pertusis, Treponema* spp.*, Mycobacterium pneumonia, Moraxella bovis, M. catarrhali*s, and possibly one for bovine Lf (bLf) in *Streptomyces uberis* (Prinz *et al.* 1999, Ling and Schryvers 2006).

LBPs from the Neisseriaceae family are the best characterized to date, consisting of 2 distinct proteins, LbpA and LbpB. These proteins are expressed from an iron-repressible operon, such that LBPs are upregulated when iron is scarce (Ling and Schryvers 2006). Indeed, the binding of hLf to cell surfaces increased 350-fold when the bacteria were grown in low iron conditions (Schryvers and Morris 1988). The Neisseriaceae LBPs are specific to hLf, binding neither bovine Lf (bLf) nor the structurally related human Tf (hTf), and bind regardless of the iron-saturation of the Lf (Schryvers and Morris 1988, Prinz *et al.* 1999).

LbpA is homologous to TonB-dependent outer-membrane proteins, with a C-terminal -barrel filled with an N-terminal plug domain (Prinz *et al.* 1999, Ling and Schryvers 2006). Most TonB systems are involved in the uptake of macromolecules, particularly iron-siderophore complexes

and vitamin B12 (Koebnik *et al.* 2005). In *Moraxella* and *Neisseria* sp., the LbpA is shown to bind two regions within the C-terminus of hLf (Wong and Schryvers 2003). The exact role of the second LBP, LbpB, has yet to be determined. *N. meningitidis* LbpB-isogenic mutants are still able to obtain iron from Lf (Bonnah and Schryvers 1998, Ling and Schryvers 2006). A possible model for LBPs and Lf interaction involves Lf binding to LbpA and LbpB, causing a conformational change in Lf that results in the release of iron and subsequent transport of the iron across the outer membrane (Ling and Schryvers 2006).

1.2 The Ability of Lactoferrin to Affect Bacterial Internalization

1.2.1 Known Affects of Lactoferrin on Bacterial Internalization

Lactoferrin is known to affect the ability of some facultative intracellular bacteria to invade host cells. Most often, Lf reduces the frequency of internalization via mechanisms that include impeding bacterial adherence to epithelial cells and degradation of bacterial protein factors necessary for invasion (Valenti and Antonini 2005), although the exact outcome differs by species and form of Lf used (Table 1.1, 1.2). The majority of research to date has focused on the affect of bLf and the N-terminal peptide Lfcin using tissue culture, but there is also evidence of Lf inhibiting bacterial invasion *in vivo* (Ajello *et al.* 2002).

However, the effect of Lf is not always so clear. For example, invasion of the Caco-2 intestinal cell line by *Listeria monocytogenes* is reportedly reduced in the presence of bLf, but no such reduction is found with *Listeria* in THP-1 macrophages (Valenti *et al.* 1999, Longhi *et al.* 2004). In contrast, bLfcin but not bLf limits internalization of *Yersinia*

spp. and *E. coli* HB101 expressing the *Yersinia* InvA protein for invasion, as well as *L. monocytogenes* (Di Biase *et al.* 2004, Longhi *et al.* 2004, Superti *et al.* 2005).

Table 1.1: Effect of lactoferrin on internalization of facultative intracellular organisms

Lf Type	Amount Tested (mg/ml)	Bacterial Species	Cell Type	Effect		Source
				-Lf	+Lf	
Human Lactoferrin	0.33	*Shigella flexneri* BS176, *Shigella flexneri*	HeLa cells		48 % inhibition [1]	Willer *et al.* 2004
		Shigella dysenteriae			48 % inhibition [1]	
		Shigella sonnei			64 % inhibition [1]	
Bovine Lactoferrin	2	*Yersinia enterocolitica*	HEp-2 cells	87 %[2]	89 %[2]	Superti *et al.* 2005
		Y. psuedotuberculosis	HEp-2 cells	62 %[2]	60 %[2]	
		E. coli HB101 (with *invA*)	HEp-2 cells	40 %[2]	38 %[2]	
	2	*E. coli* HB101 (with *invA*)	HeLa S3	34 %[2]	3.2 %[2]	Longhi *et al.* 1993
	1	Group A Streptococci	HeLa S3	0.5 %[2]	(apo-Lf) 0.012 %[2] (holo-Lf) 0.013 %[2]	Ajello *et al.* 2002
	2	Group A Streptococci	HeLa S3	0.5 %[2]	0.002 %[2]	
	1	*Listeria monocytogenes*	Caco-2 intestinal cells	3.5 %[2]	0.3 %[2]	Valenti *et al.* 1999
	0.13	*Listeria monocytogenes*	THP-1 macrophages	4.4 %[2]	4.0 %[2]	Longhi *et al.* 2004
Bovine Lactoferricin	0.5	*Yersinia enterocolitica*	HEp-2 cells	79 %[2]	7 %[2]	Di Biase *et al.* 2004
	0.5	*Yersinia psuedotuberculosis*	HEp-2 cells	75 %[2]	6 %[2]	
	0.5	*E. coli* HB101 (with *invA*)	HEp-2 cells	73 %[2]	8 %[2]	
	0.13	*Listeria monocytogenes*	THP-1 macrophages	4.4 %[2]	0.7 %[2]	Longhi *et al.* 2004

(1) Compared to internalization frequency in the non-Lf controls.
(2) Proportion of initial inoculum (as percent) recovered during gentamycin protection assay, presumed to be intracellular.

Table 1.2: Effect of lactoferrin on adhesion of facultative intracellular microorganisms

Lf Type	Amount Tested (mg/ml)	Bacterial Species	Cell Type	Effect		Source
				-LF	+LF	
Human Lactoferrin	0.33	*Shigella flexneri* BS176, *Shigella flexneri*	HeLa cells		56 % inhibition[1]	Willer *et al.* 2004
		Shigella dysentereae	HeLa cells		71 % inhibition[1]	
		Shigella sonnei	HeLa cells		51 % inhibition[1]	
Bovine Lactoferrin	2	*Yersinia enterocolitica*	HEp-2	3.3 %	3.0 %[2]	Superti *et al.* 2005
		Y. psuedotuberculosis	HEp-2	5.0 %	5.1 %[2]	
		E. coli HB101 (with invA)	HEp-2	3.8 %	3.7 %[2]	
	2	*E. coli* HB101 (with invA)	HeLa S3	12 %[2]	1.3 %[2]	Longhi *et al.* 1993
	0.13	*Listeria monocytogenes*	THP-1 macrophages		no effect	Longhi *et al.* 2004
Bovine Lactoferricin	0.5	*Yersinia enterocolitica*	HEp-2 cells	(37˚C)[3] 2.9 %[2] (28˚C)[3] 1.6 %[2]	(37˚C)[3] 28 %[2] (28˚C)[3] 1.6 %[2]	Di Biase *et al.* 2004
		Yersinia psuedotuberculosis		(37˚C)[3] 5.1 %[2] (28˚C)[3] 2.4 %[2]	(37˚C)[3] 42 %[2] (28˚C)[3] 3 %[2]	
	0.13	*Listeria monocytogenes*	THP-1 macrophages	1.6 %[2]	2.1 %[2]	Longhi *et al.* 2004

(1) Compared to adhesion frequency in the non-Lf controls.
(2) Proportion of inoculum (as percent) recovered from cells during an adhesion assay.
(3) Infection was carried out at both 37˚C, when expression of invasion-mediating proteins is low, and 28˚C when expression of invasion proteins is high.

1.2.2 Mechanism of Lactoferrin-mediated Effects on Bacterial Internalization

Many of these effects are seen at sub-inhibitory concentrations of Lf, so that a decrease in internalization is not attributed to a decrease in bacterial viability. Lf is thought to affect bacterial internalization through a number of different means that include binding to and blocking important adhesion and invasion sites, degradation of proteins necessary for bacterial adhesion and invasion and/or as an anti-microbial protein, by reducing overall bacterial survival.

Lactoferrin has been shown to affect the adhesion of microorganisms to both abiotic and cellular surfaces (Valenti and Antonini 2005). Human Lf isolated from milk inhibited adhesion of three *Shigella* species (*S. flexneri, S. dysenteriae*, and *S. sonnei*) to HeLa cells by 56-71 %, with a corresponding 50 % drop in invasion frequency (Willer *et al.* 2004). Human Lf, hLfcin, bLf, and bLfcin bind Gram-negative and Gram-positive bacterial surfaces, as well as elements of the host cell surfaces such as glycosaminoglycans (GAGs) (Valenti and Antonini 2005), potentially covering or masking bacterial receptors that act as binding sites for facultative intracellular species and thereby reducing both the ability of the bacteria to adhere and to invade (Isberg and Barnes 2001, Valenti *et al.* 2005).

This is illustrated by studies that show bovine Lfcin decreased internalization of *L. monocytogenes* in TH1-macrophages, partially attributed to competition between bLfcin and the listerial ActA surface protein for cellular binding sites (Longhi *et al.* 2004). Lactoferrin seems to have a specific effect on InvA-mediated invasion, probably due to subverting the interactions between InvA, GAGs, and eukaryotic cell surface integrins necessary for InvA-mediated invasion. Bovine Lfcin also

reduces invasion in both *Yersinia* spp. and *E. coli* HB101 transfected with the InvA gene (Longhi *et al.* 1993, Di Biase *et al.* 2004).

Lactoferrin can also directly degrade some proteins necessary for pathogen infection via proteolysis. Lactoferrin has been shown to degrade or inactivate proteins that are required for host colonization by *E. coli, S. flexneri*, and *Haemophilus influenzae* (Hendrixson *et al.* 2003, Ward *et al.* 2005). With *H. influenzae*, this activity was blocked by serine protease inhibitors, and subsequent studies have characterized a possible serine protease catalytic domain in the N-terminal region of Lf. The region is capable of cleaving arginine-rich sequences in *H. influenzae* IgA1 protease and Hap adhesins, surface proteins involved in avoiding host immune response and attachment to cellular surfaces (Hendrixson *et al.* 2003). This region is highly conserved across species, but high variability in the level of protease activity in Lf makes it difficult to judge the relative importance of proteolysis in Lf's antimicrobial properties (Valenti and Antonini 2005). Lactoferrin reduces binding of *Acintobacillus actinomycetemcomitans* to epithelial cells, probably by cleaving surface Aae proteins, homologous to the *E. coli* Hap protein (Rose *et al.* 2003).

Studies of recombinant hLf (rhLf) and *Shigella flexneri* found a reduction in internalization, but rhLf did not affect the adhesion or growth of S. *flexneri* within the time limits of the experiment (Gomez *et al.* 2003). While not affecting the host cell binding, the rhLf triggers the release and degradation of IpaB and IpaC, proteins essential for inducing bacterial uptake in epithelial cells. Likewise, bLf seems to activate the secretion of IpaB and IpaC from EIEC (Santapaola *et al.* 2004), even when bLf is separated from the bacteria with a dialysis membrane. Both *Shigella* spp. and enteroinvasive *E. coli* (EIEC) secrete virulence factors via type III

secretion systems and the low iron conditions induced by Lf may serve as a signal to modulate the secretion of virulence proteins.

Lactoferrin's effect on bacterial internalization seems to be independent of its iron-binding functions, with little to no difference in the effect of iron-saturated holo-Lf and iron-deficient apo-Lf when tested (Valenti *et al.* 1999, Ajello *et al.* 2002). Instead, sugar residues are implicated in Lf's effect on invasion efficiency. The ability of hLf to inhibit adhesion and invasion in *Shigella* spp. into HeLa cells is eliminated when fucosylated residues on the Lf are modified by treatment with sodium metaperiodate (Willer *et al.* 2004), a hypothesis supported by the observation that rhLf does not have the same effect on adhesion of *S. flexneri* to HeLa cells as hLf (Gomez *et al.* 2003, Willer *et al.* 2004).

1.2.3 Binding of Lactoferrin to Cell Surfaces

In some instances, the effect of Lf on invasive bacteria seems to be mediated by its ability to bind the eukaryotic cell surfaces. The binding of Lf to outer-membrane receptors can have a potent effect on the cellular immune response, altering the cell's reaction to infection by invasive bacteria and thus affecting the outcome of invasion. Lactoferrin is immunostimulatory, changing expression of a number of important cytokines in the presence of bacterial virulence factors (Prgomet *et al.* 2006), and has been shown to modulate apoptosis in epithelial cells during infection with invasive species (Tsai *et al.* 1999, Valenti *et al.* 1999, Superti *et al.* 2005). Furthermore, Lf's ability to bind a wide range of surface components allows it to mask binding sites necessary for intracellular bacteria to invade.

Specific receptors for hLf are widely distributed throughout different tissues in the body, with individual cell types expressing specific lactoferrin receptors (LfRs) with varying characteristics (Suzuki and Lönnerdal, 2002). The best characterized LfR was first identified in the small intestine, and is known as the SI-LFR. High levels of SI-LFR mRNA expression are found in adult tissues, in the salivary gland, heart tissue, skeletal muscle, the testes, the adrenal gland and the pancreas (Suzuki *et al.* 2005). Protein assays for LfRs in mice, based on the mouse SI-LFR homologue, show expression in the digestive tract, nervous system, stomach, reproductive system, and other tissues (Suzuki and Lönnerdal 2004). Among these tissue types Lf receptors appear to vary structurally and functionally. Some are believed to be related to iron uptake, particularly in infants, while others may regulate inflammatory response and cell maturation (Mikogami *et al.* 1995, Legrand 1997, Suzuki *et al.* 2005).

While many tissues have specific LfRs, the majority of Lf that binds cell-surfaces is believed to be interacting with negatively charged proteoglycans and nucleolin, probably through Lf's highly cationic region (Legrand *et al.* 1997). Lactoferrin can bind non-specifically to a number of receptors on cell surfaces, including receptors for low-density lipoproteins, lymphocytes, asialoglycoproteins as well as proteoglycans (Dhennin *et al.* 2000). Proteoglycan binding may facilitate the interaction between Lf and LfRs; similar to the role they play with fibroblast growth factor and its receptor (Suzuki *et al.* 2005). Though some nucleolin exists on cell surfaces as receptors, the majority is found in the nucleus and mediates cell proliferation, cell growth, cytokinesis, embryogenesis, and nucleogenesis (Legrand *et al.* 2004). Nucleolin may also play a role in Lf internalization, as it can act as an intermediary in extracellular regulation of nuclear events,

possibly even as a shuttle between the cell surface and nucleus (Legrand *et al.* 2004).

The events following Lf binding to cell surfaces have not been fully established, and seem to vary by cell type and function. In some cases, Lf has also been reported to affect cell physiology via signal transduction by simply binding surface receptors (Dhennin-Duthille *et al.* 2000). Internalization of hLf has been shown in a number of cell lines with immunofluorescence microscopy and appears to be nucleolin and proteoglycan dependent (Legrand *et al.* 2004). Lactoferrin internalization may also be clathrin-mediated, with hLf-containing vesicles found to contain markers associated with clathrin-dependent endocytosis (Legrand *et al.* 2004, Lopez *et al.* 2008). When internalization does occur, it appears to do so from the apical membrane, with fluorescent-labeled Lf internalized only from the apical side of Caco-2 cells, not the basolateral side (Ashida *et al.* 2004, Mulligan *et al.* 2006).

1.3 The Ability of Lactoferrin to Interact with DNA

1.3.1 Nuclear Localization of Lactoferrin and DNA Binding

Once Lf binds epithelial cells, it can be translocated into the cytoplasm. Moreover a proportion of the internalized Lf also enters the nucleus (Briggs *et al.* 1981, Fleet 1995, Penco *et al.* 2001, Haverson *et al.* 2002, Ashida *et al.* 2004, Legrand *et al.* 2004, Mariller *et al.* 2007, Lopez *et al.* 2008). Nuclear accumulation is rapid and directed by a nuclear localization signal (NLS), believed to be a short stretch of arginine residues in the N-terminus (Gly-Arg-Arg-Arg-Arg (GRRRR)) with similarity to the NLS of ribosomal proteins and nuclear signals in a number of viruses

(Penco *et al.* 2001). This same region is essential for hLf interactions with heparin, LPS, lysozyme, and DNA (van Berkel *et al.* 1997).

Lactoferrin binds to single stranded and double stranded DNA, preferentially binding three major DNA consensus sequences known as lactoferrin response elements (LFRE) (Fleet 1995). These include LFRE1 (GGCACTT (G/A) C), LFRE2 (TAGA (A/G) GATCAA), and LFRE3 (ACTACAGTCTACA) (He and Furmanski 1995). Studies have shown that each of the three main LFREs is able stimulate transcription (Fleet 1995, He and Furmanski 1995, Son *et al.* 2002, Mariller *et al.* 2007). Single base pair changes greatly reduce the amount of transcription activation, and no activation is found when LFRE1 is scrambled to provide a sequence of the same length and base composition, suggesting that the interaction is specific (He and Furmanski 1995). Other LFREs have since been identified, and most are similar to one these three.

Lactoferrin appears to have two binding sites that can interact with DNA, both specifically and non-specifically. On the N-terminus, the amino acids Gly-Arg-Arg-Arg-Arg (GRRRRR) have been implicated, with binding efficiency of Lf to DNA reduced by 66 % with removal of the first three amino acids and by approximately 95 % with removal of all five N-terminal amino acids (van Berkel *et al.* 1997). The second binding site, probably on the C-terminal lobe of Lf, has a much lower affinity to DNA, binding LFREs with 1250-fold reduced affinity than the N-terminal region (Kanyshkova, *et al.* 1999). Computer modeling has suggested that the groove between the C-terminal and N-terminal lobes could also interact with DNA (Mariller *et al.* 2007). DNA binding seems to occur slightly more with apo-Lf than holo-Lf, though some studies have found the iron-saturation of Lf to be unimportant (He and Furmanski 1995, van Berkel *et al.* 1997, Kanyshkova *et al.* 1999).

1.3.2 The Effect of Lactoferrin on Eukaryotic Gene Expression

As suggested by nuclear localization and DNA binding ability, Lf has been shown to act on the expression of a number of genes, particularly genes involved in host immune response and cell division. However, it is unclear if the effect of Lf is due to direct action on specific gene promoters or occurring through some intermediary signaling. Lactoferrin treatment increases the tyrosine phosphorylation of numerous intracellular polypeptides involved in signal transduction in cultured cells, suggesting an intermediary role in modulating gene expression (Dhennin-Duthille *et al.* 2000) but, despite many studies, there is still no general consensus on how Lf affects gene expression.

A broad range of research has been done with Lf and the expression of cytokines, signaling molecules involved in immunomodulation. Lf is capable of binding unmethylated CpG dinucleotides found in bacterial genomes and bacterial LPS, both potent stimulators of host immune response that acts as a signal of bacterial infection. The addition of Lf to CpG or LPS stimulated cells has been shown to affect expression of both pro-inflammatory and anti-inflammatory cytokines in different experimental approaches (Britigain *et al.* 2001, Haverson *et al.* 2002, Son *et al.* 2002, Mulligan *et al.* 2006, Prgomet *et al.* 2006). Lf can also affect expression of genes involved in cell division and differentiation. Treatment with Lf arrested breast cancer cells at the G1 and S transition in the cell cycle by changing the expression and activity of a number of cell cycle progression regulators (Damiens *et al.* 1998).

Lf has been shown to affect expression of the KDR/Flk-1 receptor, which binds extracellular mitogens that induce angiogenesis (Kim *et al.* 2006). As with most of the changes in protein expression associated with Lf, it is unknown of the KDR/Flk-1 upregulation is due to direct gene activation by Lf or is via other regulatory paths, such as MAP kinase. In lymphocytes, Lf treatment induces a rapid and transient increase in the activity of the mitogen activated protein (MAP) kinase, a key regulatory enzyme involved in the differentiation and proliferation process (Dhennin-Duthille *et al.* 2000). Similarly, much of the affect of Lf on cytokine expression in CpG and LPS stimulated cells is probably due to its binding of extracellular CpG-ODN and LPS and thus interfere with how they interact with cell receptors.

There are, however, two cases of Lf directly activating transcription of genes. Five putative LFREs were identified in the 5' flanking region of the gene for the human cytokine IL-1, occurring between –3203 and –1043 of the gene (Son *et al.* 2002). All are identical or similar to the LFRE1 identified by He and Furmanski. Gel mobility shift assays confirm that Lf binds to all five sites, although the combination of sites sufficient for activation is unknown (Son *et al.* 2002). IL-1 affects the expression of other proteins, and Lf's effect on IL-1 is probably responsible for some of the changes in expression seen in other proteins, particularly granulocyte macrophage colony-stimulating factor and possibly tracheal antimicrobial peptide (Penco *et al.* 1995, Velliyagounder *et al.* 2003).

In the second case, upregulation of a cytoplasmic isoform of Lf, delta lactoferrin (Lf), has been shown to lead to cell cycle arrest in the S phase (Mariller *et al.* 2007). Micro-array analysis shows that increased levels of Lf doubles transcription of the gene *Skp1,* a protein involved in

the regulation of protein degradation critical for normal G_1- and S-phase progression in the cell cycle (Mariller *et al.* 2007).

Two sequences similar to LFRE1 and LFRE2 were found within the Skp1 promoter. When placed before a luciferase gene construct, both were able to drive expression in the presence of Lf (Mariller *et al.* 2007). As the two sequences appear to act synergistically, there is some evidence that Lf may bind both sequences as part of the same complex, perhaps with both the N-terminal and C-terminal DNA binding domains (Mariller *et al.* 2007). While normally cytoplasmic, Lf was shown to localize to the nucleus, further supporting its role in *skp1* transcription (Mariller *et al.* 2007). While exogenous Lf can be endocytized and brought into the human nucleus, it is possible that cyoplasmic forms of Lf are more likely to directly activate genes, with external Lf liable to induce activation via secondary messengers.

1.3.3 The Ability of Lactoferrin to act as a DNA Vector

Lactoferrin's ability to bind DNA and be internalized into the nucleus of cells makes it a potential DNA vector and it has already been studied as prospective agent for gene therapy and drug delivery. Lactoferrin has been used as a nuclear localizing agent for polyethylenimine (PEI). This agent is capable of binding and compacting plasmid DNA and protecting it from nuclease degradation (Elfinger *et al.* 2007) however the use of PEI for DNA delivery is limited because of macrophage clearance before it enters epithelial cells. Fluorescence labeled Lf was able to bind to a PEI-luciferase construct and bring it into human bronchial cell lines (Elfinger *et al.* 2007). Expression of the luciferase gene suggests it was translocated to the nucleus.

Lactoferrin is also able to direct the internalization of stabilized plasmid lipid particles (SPLPs), designed for *in vivo* delivery of plasmid DNA, although failure to detect subsequent gene expression in an animal model suggests further work needs to be done with Lf as a targeting agent (Bartsch *et al.* 2005). Another area of investigation is focusing on the use of Lf as a nuclear localization factor for peptide-nucleic acids (PNA), which have a peptide backbone but contain base pairs and can mimic DNA sequences, giving them the potential to act as transcription factor decoys, reducing transcription of homologous DNA sequences by attracting transcription factors that would otherwise drive gene expression (Gambari 2004). The N-terminal fragment of Lf containing the nuclear localization signal is sufficient to deliver PNA to the nucleus (Penco 2001, Gambari 2004).

Some interesting work has been done that shows intact Lf directly binding DNA and delivering it *in vivo*. Lf was bound to two different plasmids, and the plasmid-bound Lf was injected into the muscles of mice, which were then monitored for gene expression (Sinogeeva *et al.* 2000). Gene products from both plasmids were detected at the site of injection, and one carrying the human dystrophin gene was found in the muscles of other limbs, suggesting that the Lf had been able to broadly deliver the human dystrophin gene (Sinogeeva *et al.* 2000).

The Use of *Helicobacter pylori* as a Model Organism to Investigate Interactions between Lactoferrin and Bacteria

We began with the hypothesis that bacteria that regularly colonize host surfaces with large quantities of Lf would need to adapt to Lf's antimicrobial properties. Those that adapt might even exploit Lf (Heinemann 2008). This is interesting as it applies both to the microbial ecology of the human body, and to issues of biosafety, with the potential mass production of human Lf outside of the human body. *Helicobacter pylori* was chosen as a model organism because it lives in a high-Lf environment and some research suggests it is able to use Lf as an iron-source (Husson *et al.* 1995, Dhaenens *et al.* 1997, Velayudhan *et al.* 2000)

H. pylori is a spiral shaped, Gram-negative bacterium. Infection with *H. pylori* leads to chronic gastritis in the host, and in some cases can lead to the development of peptic ulcers and gastric cancer (Blaser 1998, Huang *et al.* 1998, Rothenbacher and Hermann 2003). This species has colonized the human stomach for tens of thousands of years, and is highly adapted to its environment (Falush *et al.* 2003, Blaser and Atherton 2004, Kusters 2006). These adaptions include production of a stomach acid neutralizing enzyme (urease), flagella to move through the mucous layer of the stomach, and the virulence factors cytoxin associated gene A (CagA) and vacuolating cytotoxin A (VacA) (Blaser and Atherton 2004, Bourzac and Guilleman 2005, Kusters 2006). Production of CagA in particular is associated with an increased risk of developing ulcers and gastric cancer disease, and is used to distinguish between more pathogenic Type I strains (cagA+) and Type II strains (cagA-) (Blaser and Atherton 2004, Kusters 2006).

Some research has suggested that *H. pylori* has also evolved to utilize hLf (Husson *et al.* 1993, Dhaenens *et al.* 1997), which is abundant on the mucosal surface of the stomach. There is a lack of confirmatory studies on if and how *H. pylori* uses hLf as an iron-source, and how hLf may be affecting other aspects of *H. pylori*'s biology.

1.4 The Ability of *H. pylori* to use Lactoferrin as an Iron Source

1.4.1 Non-Lactoferrin Sources of Iron for H. pylori

H. pylori is thought to encounter immense variation in the source and concentration of bioavailable iron. The mucous layer of the stomach epithelium is an Lf- rich environment, which can lead to iron scarcity (Ling and Schryvers 2006). Yet there is the potential for significant influxes of iron released from food by peptic degradation, or acquired directly from gastric epithelial cells as a result of the *H. pylori*-mediated inflammatory response (van Vliet *et al.* 2002). To further complicate the situation, the exploitability of the iron itself depends on its oxidative state, which is affected by changing pH in the stomach (vanVliet *et al.* 2002).

H. pylori has 13 genes encoding putative elements of iron-transport and iron storage systems (Berg *et al.* 1997, Tomb *et al.* 1997, Alm *et al.* 1999). Many of these genes are regulated by the ferric uptake regulator Fur, a transcriptional repressor that generally acts to down regulate iron-uptake systems when iron is abundant. While Fur is best known for its role in controlling iron uptake in bacteria, in *H. pylori* Fur also regulates intracellular iron storage, modulates urease expression in response to nickel and may play a role in the ability of *H. pylori* to survive the acidic

environment of the stomach (Delany *et al.* 2001, Bijlsma *et al.* 2002, van Vliet *et al.* 2002).

H. pylori also has homologues to membrane-bound, iron-transport proteins that in other bacteria allow the uptake of free iron in the environment. *H. pylori* can take up the more extracellularly rare Fe(II) via Feo, a cytoplasmic-membrane-bound, iron permease found in many bacteria (Velayudhan *et al.* 2000). This system may be particularly important in conditions of low-oxygen such as is found in the stomach, where one would expect a higher ratio of Fe(II) to Fe(III) (Andrews *et al.* 2003). Additionally, the genome of *H. pylori* encodes 3 homologues to the FecA protein, which is a Fe(III)-dicitrate transporter although their role in iron-uptake remains unclear (Berg *et al.* 1997, Velayudhan *et al.* 2000).

H. pylori may increase its ability to extract iron from the environment by excreting iron-binding proteins, such as siderophores, or obtaining iron directly off of host proteins, such as heme and Lf. Siderophores are a group of highly divergent, high-affinity iron chelators that are produced and excreted from organisms with the purpose of binding extracellular Fe(III) in the environment that can then be brought back into the cell (Meithke and Maraheil 2007). Most studies have reported no siderophore production in *H. pylori*, and indeed in none of the gastric *Helicobacter* species (Husson *et al.* 1993, Illingworth *et al.* 1993, Dhaenens *et al.* 1999). The one report of *H. pylori* siderophore production may instead be attributable to the extracellular ferric reductase activity associated with the synthesis and excretion of riboflavin (Worst *et al.* 1998, vanVliet *et al.* 2002).

Many pathogenic bacteria have evolved the means to acquire iron from heme, the most abundant source of iron in the body. Heme and hemoglobin must be released from red blood cells before extracellular

pathogens have access to it, usually through the use of haemolysins and proteases (Andrew *et al.* 2003). *H. pylori* has been reported to use heme as an iron source in iron-limited media and when other iron-uptake systems have been knocked out (Husson *et al.* 1993, Velayudhan *et al.* 2000) via three high affinity, outer membrane heme binding proteins (Worst *et al.* 1995).

1.4.2 Human Lactoferrin as an Iron Source for H. pylori

The data on *H. pylori*'s use of lactoferrin as an iron source is mixed. Evidence that *H. pylori* has a putative LBP and can use hLf as an iron-source is offset by at least one study that reports no use of hLf as well as unrelated studies demonstrating that certain forms of Lf inhibit *H. pylori* growth (Husson *et al.* 1993, Illingworth *et al.* 1993, Dhaenens *et al.* 1999, Opekun *et al.* 1999).

H. pylori lives in a particularly high Lf environment, as infection causes inflammation of the gut, which in turn increases the release of Lf. Lactoferrin is among the genes upregulated in epithelial cells during *H. pylori* infection and studies have found a positive correlation between the degree of *H. pylori* induced gastric inflammation and the concentration of Lf in the gastric mucosa (Wen *et al.* 2004, Choe *et al.* 2003). One group found that Lf expression in the stomach was highest when the patient had both *H. pylori* infection and iron-deficiency anemia, which is widely associated with *H. pylori* infection in adolescents (Choe *et al.* 2003). These researchers hypothesized that *H. pylori*'s use of Lf-bound iron in the stomach may be a contributing factor to the host's inability to acquire sufficient iron leading to subsequent anemia.

Husson *et al.* found that partially iron-saturated hLf could support full growth of *H. pylori* strain 43504 and 15 clinical isolates in iron-limited media (Husson *et al.* 1993). The bacteria were unable to grow when the medium was supplemented with bLf or hTf. This suggests that the mechanism, similar to those described for other bacteria such as *N. meningitidis*, is specific to host Lf (Husson *et al.* 1993). Additionally, *feoB* mutants unable to get Fe(II) from the environment were able to survive in iron-limited conditions by using iron from human Lf and Tf (Velayudhan *et al.* 2000).

Husson's study also showed that *H. pylori* were unable to recover growth when the Lf was separated from the bacteria with a dialysis bag, indicating that *H. pylori*'s use of hLf may be dependent on cell-to-protein contact (Husson *et al.* 1993). A 70 kDa outer membrane protein of *H. pylori* was later identified as a putative LBP, shown via affinity chromatography to bind biotinylated-hLf (Dhaenens *et al.* 1997). The specificity of this putative LBP was examined via competitive binding experiments, and Lf binding was shown to be uninhibited by horse Tf, bovine Tf, and hTf (Dhaenens *et al.* 1997). Partial inhibition was observed with bLf but the bacteria were unable to use it for growth in iron-limited conditions. These findings suggest that the *H. pylori* LBP, which is only expressed in iron-limited medium, may be involved in removing iron from Lf (Dhaenens *et al.* 1997). However, further characterization of the protein has not occurred and the corresponding gene has yet to be identified.

1.4.3 Lactoferrin as a Therapeutic Against H. pylori

Some forms of Lf are under investigation for their ability to inhibit *H. pylori* growth, particularly as possible adjuvants to antibiotic therapy.

Lactoferrin is shown to work as a therapeutic against many microorganisms (Arnold *et al.* 1980). Some authors have reported that bLf has a bacteriostatic effect on *H. pylori in vitro*, which the authors attribute to iron sequestering (Dial *et al.* 1998). This is consistent with earlier work suggesting that *H. pylori* is unable to use iron from bLf for growth (Husson *et al.* 1993, Dhaenens *et al.* 1997). Bovine Lf has also been tested *in vivo* as a potential therapeutic, but success has been mixed (Di Biase *et al.* 2006, Zullo *et al.* 2007).

More interesting, perhaps, is the use of rhLf as a potential therapeutic agent. Miehlke *et al.* found recombinant hLf (rhLf), reduced the growth of 8 of 13 clinical isolates (Miehlke *et al.* 1996, Joshi *et al.* 2001). Methodological issues have been raised with the study, though, and work investigating rhLf's potential to act a therapeutic have not found it successful (Opekun *et al.* 1999, Guttner *et al.* 2003). The possible ability of rhLf to impeded *H. pylori* growth is not necessarily inconsistent with the ability of *H. pylori* to use hLf as an iron source. It is possible that rhLf could affect *H. pylori* differently than hLf, as it would be expected to have differing patterns and types of post translational modifications, such as glycosylation, depending on the species in which it is produced. Indeed rhLf has been shown to have different impacts on the physiology of *Shigella* spp. compared to hLf (Gomez *et al.* 2003).

1.5 The Ability of *H. pylori* to be Internalized into Epithelial Cells

1.5.1 Evidence for Internalization of H. pylori into Epithelial Cells

There is considerable controversy over whether *H. pylori* should be considered a facultative intracellular microorganism (Peterson and Krogfelt 2003, Dubois and Boren 2007). The evidence we do have comes mostly from *in vitro* studies with cultured cell lines. Taken together, these studies suggest that *H. pylori* internalization occurs, and is an active, host-cell mediated process with the bacteria surviving in vacuoles within epithelial cells (Su *et al.* 1999, Amieva *et al.* 2002. As such, there is a growing body of *in vitro* evidence that suggests that *H. pylori* is invasive under certain conditions, but whether this internalization is biologically relevant remains unclear due to scarce evidence *in vivo*.

Recent work using immunohistochemistry and fluorescence *in situ* hybridization provides evidence of intracellular *H. pylori* in the lamina propria, within gastric epithelial cells, and immunocytes of patients with gastric diseases (Ogata 1997, Oh *et al.* 2005, Dubois and Boren 2007, Necchi *et al.* 2007). Moreover, some of these bacteria were still able to produce mRNA and antigens, suggesting they were viable (Necchi *et al.* 2007). Yet, while internalization does seem to occur *in vivo*, it does so at much lower frequencies than have been reported from *in vitro* studies and some studies of clinical biopsies and primary cultures of human antral gastric epithelial cells have failed to find evidence of internalization (Peterson and Krogfelt 2003). More often, the presence of intracellular bacteria is confirmed but the frequency of invasion remains quite low. Ko *et al.* found intracellular bacteria in only 2 of 100 samples of gastric antral biopsy specimens immuno-stained for *H. pylori* (Ko *et al.* 1999). In

another study, intracellular *H. pylori* was found in four out of eight patients with either gastric ulcer or chronic gastritis, but only in 1 % of all examined cells (Peterson and Krogfelt 2003).

In such instances where intracellular *H. pylori* are found *in vivo*, the frequency of invasion seems to be higher in damaged epithelial cells and around active ulcers. In a study of 144 gastric biopsies using light and differential interference contrast microscopy, 5.6 % of patients with minor epithelial damage had internalized *H. pylori*, increasing to 100 % in patients with severe epithelial damage (Chan 1992). However, it remains to be determined if the internalized bacteria were in some way responsible for the increased tissue damage, suggesting a role for invasion in *H. pylori* pathogenesis, or if they were simply more able to invade damaged tissue.

In vitro work using immortalized cancer cell lines has provided a more robust pool of evidence. The proportion of infecting bacteria that are engulfed by host cells during the widely used gentamycin (GM) protection assay range across the literature from rare (less than 0.0019 %; Wilkinson *et al.* 1998) to relatively frequent (15 %; Peterson *et al.* 2000), varying according to *H. pylori* strain and cell type used (Table 1.3). Generally, frequencies seem to be higher for Type I strains of *H. pylori,* defined as having the CagA pathogenicity island that plays an important role in *H. pylori* pathogenesis (Kusters *et al.* 2006), than the Type II strains, which lack it. Among cell types, the highest frequencies of internalization have been reported for the gastric adenocarcinoma cell lines AGS and Kato III, with lower frequencies of internalization found in laryngeal adenocarcinoma HEp-2 cell lines and no significant internalization found in assays with cervical adenocarcincoma cell line HeLa (Rautelin *et al.* 1995, Peterson *et al.* 2001, Peterson and Krogfelt 2003). This degree of variation in reported invasion frequencies among strains and cell types

suggests that both strain-specific and cell-specific factors may mediate the internalization of *H. pylori* into cultured cell lines.

Table 1.3: Frequencies of *H. pylori* internalization into epithelial cell lines using gentamycin protection assays

Strain	Cell Type	Internalization Frequency[1]	Source
Type I			
AF4	AGS	~ 0.006 %	Peterson *et al.* 2001
G27	AGS	~ 0.055 %	Peterson *et al.* 2001
G27	AGS	1.0 %	Amieva *et al.* 2002
P119	AGS	1.5 %	Su *et al.* 1999
A5	AGS	2.0 %	Su *et al.* 1999
26695[2]	AGS	2.5 %	Kwok *et al.* 2002
AF4	AGS	15.0 %	Peterson *et al.* 2000
Type II			
51932	AGS	0.0005 %	Peterson *et al.* 2001
M019	AGS	1.0 %	Su *et al.* 1999
51934	AGS	8.0 %	Peterson *et al.* 2000
Un-typed			
Various clinical isolates	HEp-2	0.0006 %-0.0019 %	Wilkinson *et al.* 1998

1. Proportion of inoculum (as percent) that survived the gentamycin protection assay; presumed to be intracellular.
2. Strain is +*cagA*, but gene product may not be functional. Type is questionable.

1.5.2 Mechanism of Internalization by H. pylori

Most *in vitro* work suggests that *H. pylori* internalization is an active, host-cell mediated process. There is evidence that the process is mediated by binding integrins, cell surface receptors that are well known to promote bacterial internalization (Su *et al.* 1999, Scibelli *et al.* 2007). *H. pylori* internalization has also been associated with localized tyrosine phosphatase signals and condensed actin filaments, suggesting signal-induced uptake (Kwok *et al.* 2002). Further evidence from HEp-2 cells

supports this, with a 100-fold reduction in invasion in the presence of ammonium chloride, which inhibits receptor-mediated endocytosis, and by dansylcadaverine, which inhibits receptor clustering and internalization (Peterson and Krogfelt 2003).

While there has been some contradictory evidence with non-gastric cell lines, most studies utilizing AGS cells support the role of actin-mediated processes in *H. pylori* internalization (Su *et al.* 1999, Peterson *et al.* 2001, Amieva *et al.* 2002). Similar processes are important for the internalization of many invasive bacteria (Kwok *et al.* 2002). Multiple experiments, including some using video microscopy, have shown that cytochalasin D, which abrogates actin polymerization, can reduce or eliminate *H. pylori* invasion (Amieva *et al.* 2002, Peterson and Krogfelt 2003). Using a scanning electron microscope, Kwok *et al.* found evidence to support *H. pylori* entering AGS cells via zipper-like, receptor-mediated endocytosis, similar to that described for *Yersinia, Neisseria*, *Listeria* and *Streptococcus* spp. (Kwok *et al.* 2002). The mechanism involves the host cell membrane engulfing the bacteria at the site of attachment to such an extent that the membrane zips up around the entire surface of the bacterium.

1.5.3 Intracellular Survival of H. pylori

After internalization, endocytosed particles are generally targeted to lysosomes, which contain hydrolytic enzymes and antimicrobial agents. Facultative intracellular bacteria regularly subvert the host cell endocytotic pathways to generate a safe intracellular niche. They do so with a variety of mechanisms, often escaping into the cytoplasm, avoiding fusion with lysosomes, or neutralizing the bioactive components within lysosomes

after fusion (Terebiznik *et al.* 2006). Through live imaging and differential interference contrast microscopy, *H. pylori* have been seen to survive engulfment, with live, moving bacteria recorded in cellular vacuoles o up to 8 hrs after infection (Amieva *et al.* 2002). Significant numbers have been recovered from GM protection assays as much as 48 hrs post-infection (Terebiznik *et al.* 2006). As there is little evidence of *H. pylori* living free in the cell cytoplasm, it probably survives by bypassing or surviving lysosomal fusion (Amieva *et al.* 2002, Peterson and Krogfelt 2003).

H. pylori-containing vacuoles possess markers of late endosomes, which typically fuse with lysosomes, after 24 hrs of invasion (Terebiznik *et al.* 2006). Fluorescent probes loaded into lysosomes pre-infection were also found in *H. pylori* containing vacuoles post-infection, further evidence of lysosomal fusion (Terebiznik *et al.* 2006). To survive this fusion, *H. pylori* require a mechanism(s) to inactivate the bactericidal properties of lysosomes because even though they reside in the stomach, *H. pylori* are not acidophilic. Reducing the acidity in lysosomes could be important for their survival, though the evidence for them doing so is mixed (Amieva *et al.* 2002, Terebiznik *et al.* 2006). Neutralization of the toxic components in lysosomes may also include inactivation of bactericidal Cathepsin D, which appears to be less concentrated in *H. pylori*-containing vacuoles (Terebiznik, *et al.* 2006). Levels of Cathepsin D were lowest in vacuoles containing strains of *H. pylori* with the gene for the vacuolating cytotoxin VacA, which has been implicated in intracellular survival in a number of studies.

Whereas all *H. pylori* strains possess the *vacA* gene, only 50 % of strains express the VacA cytotoxin (Atherton, 1995). VacA induces extensive vacuolation in cultured cells *in vitro*, and has an important role in

H. pylori pathogenesis (Kusters *et al.* 2006). Moreover, the presence of VacA seems to promote the generation of larger vacuolar compartments within the cell and mediate fusion among *H. pylori*-containing vacuoles (Terebiznik *et al.* 2006). VacA isogenic mutants do not seem to differ in their ability to adhere to or invade TC cells (Peterson *et al.* 2001, Amieva *et al.* 2002), but tend to not survive as long within the host cell, which is consistent with VacA's ability to modulate the vacuolar environment. Amieva *et al.* found evidence that VacA-negative, isogenic mutants survived 13 hrs less than VacA-positive wild type (WT) strains, and Peterson *et al.* found intracellular survival among VacA-negative mutants to be about 7.5 % of the WT at 24 hrs post-infection (Peterson *et al.* 2001, Amieva *et al.* 2002). The effect was reversible with the addition of broth culture filtrates and purified VacA toxin from the VacA positive strains (Peterson *et al.* 2001, Terebiznik *et al.* 2006).

Whereas some facultative intracellular bacteria are able to replicate within the host cells, the total number of live *H. pylori* recovered from *in vitro* assays slowly decreases over time (Wilkonson *et al.* 1998, Peterson *et al.* 2001). There is evidence to suggest that this may relate to bacteria being released back into the extracellular environment (Amieva *et al.* 2002). The potential for *H. pylori* to recolonize the extracellular environment has important implications for *H. pylori* pathogenesis. *H. pylori* is difficult to treat with antibiotics, with infection frequently reoccurring after antibiotic therapy has ceased. Invasion can be a way of evading host defenses and antibiotics, with host cells serving as a reservoir of bacteria for re-infection. To counter-act this, most current treatment regimens include the use of macrolides, which can concentrate intracellularly (Pechere 2001).

The majority of information we have from *H. pylori* internalization comes from *in vitro* work, and the difference between the prevalence of *H. pylori* internalization in *in vivo* and *in vitro* studies is notable. This difference may be reconciled with future work that more precisely replicates *in vivo* conditions *in vitro* (Peterson and Krogfelt 2003). Also further *in vivo* studies using *H. pylori* specific markers may corroborate *in vitro* work on the mechanisms by which *H. pylori* invade and survive inside epithelial cells. The question at this point is not so much whether *H. pylori* has the ability to invade, but whether the *in vitro* observations hold true *in vivo*, and whether invasion has any affect on pathogenesis or is just incidental.

Objectives of Current Study

The aim of this study was to increase understanding of how Lf interacts with bacteria, especially ones that regularly come into contact with it, and to further our knowledge of the microbial ecology of the human body. To do this, we have chosen *H. pylori* as a model organism

While Lf impedes the growth of most bacteria, some human pathogens are not inhibited by Lf and can even utilize Lf-bound iron for growth. *H. pylori* may be one of these species (Husson *et al.* 1993, Velayudhan *et al.* 2000), potentially expressing an Lf binding protein capable of specifically interacting with hLf (Dhaenens *et al.* 1997). Most characterized LBPs in prokaryotes have been studied in relation to their ability to use Lf as an iron source. Yet many of these species have redundant mechanisms for acquiring iron, and in some, LBPs do not appear to play an important role in iron-acquisition (Ling and Schryvers 2006). Furthermore, *Neisseriaceae* LBPs bind Lf regardless of iron

saturation, though binding does increase when iron is scarce (Ling and Schryvers 2006). LBPs fill a variety of functions in eukaryotes, and the putative role of LBPs for iron-acquisition in bacteria does not preclude additional, as yet unidentified, functions.

For example, Lf has been shown to affect the internalization of a number of facultative intracellular bacteria. This is done either through binding elements necessary for bacterial adhesion to epithelial cells, or by degrading bacterial invasion factors (Valenti and Antonini 2005). To date, however, little work has been done on species that are adapted to high levels of Lf and potentially express LBPs. Lactoferrin may have little effect on the invasion of these species, or, because hLf also binds and is internalized into epithelial cells, these bacteria may able to invade at a higher frequency by directly binding hLf bound to the cell surface.

It is also possible that Lf could be internalized into bacterial cells as it is with eukaryotic cells, and thus come into contact with bacterial DNA. Given that one of Lf's many functions in human cells is its ability to act as a transcription factor (Fleet 1995, He and Furmanski 1995), the possibility exists that Lf may also be able to act as a transcription factor in prokaryotes. *In vitro* work has shown that Lf binds three distinct DNA sequences known as Lf response elements (LFRE) with a high affinity and specificity, and can increase expression of reporter genes with LFREs in their promoter region (He and Furmanski 1995, Fleet 1995). These elements have also been found in the promoter of the two human genes known to be directly activated by Lf (Son *et al.* 2002, Mariller *et al.* 2007). A presence Lf binding sites in bacterial genomes would suggest possible functional roles for Lf.

In addition, Lf's ability to bind DNA makes it a potential DNA vector for horizontal gene transfer, which is the movement of genes

between species. This is an especially important issue for genetically modified organisms (GMOs), and the potential for transgenes to move into other, non-GMO species is the focus around which much risk assessment revolves. Genes reportedly transfer between bacteria within human cells (Ferguson *et al.* 2002). Thus, by affecting invasion frequencies, Lf may affect horizontal gene transfer between bacteria, and between bacteria and human cells.

The aims of this study were:

- To confirm the ability of *H. pylori* to utilize Lf as an iron-source in iron limited media (Chapter 2).
- To determine if Lf affects the generally low frequency of internalization of *H. pylori* into gastric epithelial cells (Chapter 3).
- To search for the possible existence of Lf binding sites in a wide array of bacteria. *H. pylori* was examined specifically to determine if any LFREs occur in locations that suggest functional roles for Lf (Chapter 4).

Chapter 2:
The Ability of *Helicobacter pylori* to Utilize Lactoferrin as an Iron-source when Iron is Limited.

2.1 Experimental Justification

Lactoferrin binds iron on mucosal surfaces of the body, limiting the free iron available to bacteria. Pathogenic bacteria have various means to acquire iron in the host, and some are able to liberate iron from host iron-binding proteins such as Lf. *H. pylori* have been reported to use hLf as an iron-source and express a 70 kDa lactoferrin binding protein (LBP) (Husson *et al.* 1993, Dhaenens *et al.* 1997). This use appears to be hLf-specific, because bLf and even recombinant hLf (rhLf) are considered as potential therapeutic agents against *H. pylori* (Miehlke *et al.* 1996, Opekun *et al.* 1999, Di Biase *et al.* 2006, Zullo *et al.* 2007). The ability of *H. pylori* to use Lf has not been widely researched and the mechanism by which it may do so remains unclear.

Here, the ability of type strains of *H. pylori* to use Lf as an iron-source was tested. A system similar to that of Husson *et al.* was used, where *H. pylori* was grown in iron-limited medium supplemented with hLf as a potential iron source (Husson *et al.* 1993). Iron-limiting conditions were created in this study by the addition of desferioxamine (DE), a potent iron-chelator, to *H. pylori* growth medium. The "iron-limited medium" was then supplemented with Lf from human milk to see if growth recovered. Our hypothesis was that if *H. pylori* growth is not inhibited by hLf, then hLf would not reduce growth in iron-replete or iron-limited conditions; and if *H. pylori* could use hLf as an iron source, then the addition of Lf to iron-limited medium should result in growth recovery. *H. pylori* can grow with $FeCl_3$ as a sole iron-source, taking up Fe(III) via the

FeoB iron-acquisition system and possibly other means (Velayudhan *et al.* 2000), and therefore $FeCl_3$ was used as a positive control.

2.2 Materials and Methods

2.2.1 Bacterial Strains and Culture Conditions

Three well-characterized *H. pylori* type strains were used for this research: 60190 (*cagA*+ / *vacA* s1m1), Tx30a (*cagA*- / *vacA* s2m2), and the mouse-adapted strain SS1 (*cagA*+/ *vacA* s2m2) (Cover *et al.* 1990, Atherton *et al.* 1995, Lee *et al*. 1997). Cultures were maintained on Colombia blood agar plates (Fort Richard, NZ; see Appendix I), and grown for assay in culture broth consisting of 2.8 % (w/v) Brucella Broth (BB; BD) supplemented with 5 % fetal bovine serum (FBS; Gibco). Cultures were incubated at 37˚C in 10 % CO_2. Master stocks were stored at –70˚C in glycerol storage medium. Iron-replete medium was standard BB + 5 % FBS (defined above), and iron-limited medium was BB + 5 % FBS supplemented with 20 μM of the iron chelater desferoxamine mesylate (DE) (Sigma) unless otherwise stated.

2.2.2 Bacterial Growth Assays

To assess the affect of Lf on the growth of *H. pylori*, bacteria were grown in iron-replete or iron-limited broth culture, supplemented with either $FeCl_3$, ~25 % iron-saturated Lf from human milk (Biochemika) or ~85 % iron-saturated (holo) Lf (Biochemika) as potential iron sources. Growth was measured as the optical density at 650nm (OD_{650}) on a SpectroMax190 spectrophotometer (MDS).

Before starting the bacterial growth assays, the minimal concentration of DE capable of reducing growth was determined for each of the three *H. pylori* strains used in this study. Strain 60190 was used as a standard, and all experiments were done in triplicate. Strains SS1 and Tx30a were tested to confirm they displayed a similar growth pattern in differing concentrations of DE. These assays were carried out using iron-replete medium (BB, 5 % FBS), supplemented with DE ranging from 10

μM to 50 μM (final concentration). Additionally, growth was also measured for each concentration of DE in the presence of 10 μM $FeCl_3$, to determine the ability of iron supplementation to overcome the iron-chelating effect of DE.

To determine if *H. pylori* can utilize Lf as an iron-source, bacteria were grown in iron-limited medium supplemented with 0.5mg/ml or 1 mg/ml of partially iron-saturated Lf (binding ~3 μM iron and ~6 μM iron respectively), or 0.5 mg/ml of fully iron-saturated iron (binding ~10 μM iron). Controls included bacterial growth in iron-replete medium (positive), iron-limited medium, and iron-limited medium supplemented with 10 μM $FeCl_3$. Bacteria were also cultured in iron-replete medium in the presence of hLF.

For all assays, 2 ml of each condition was prepared and aliquoted into 3 wells (200μl each) of a 96 well plate (BD) for a no-bacteria, absorbance control. *H. pylori* were added to the remainder of each condition (approximately 5 $x10^6$ bacteria per ml from an overnight broth culture), which was then aliquoted into 5 test wells (200μl each).

Culture absorbance was measured (OD_{650}) at 0, 18, 24, 42, 48, 66 and 72 hrs. Between measurements, the 96-well plates were incubated on a rotary shaker (120 rpm) at 37˚C in 10 % CO_2. Growth was calculated as average OD_{650} of the wells with bacteria, minus the average OD_{650} of the no-bacteria controls. Growth in each condition was normalized to the iron-replete positive control. Statistical significance between conditions was determined with a Student t-test

2.2.3 SDS Page and Silver Staining

To detect phenotypic changes in *H. pylori* grown in iron-limiting conditions, bacterial cell lysates were run on an SDS acrylamide gel and proteins were silver-stained. Bacteria were grown in iron-replete and iron-

limited culture conditions, using both Brucella broth with 5 % FBS and F12 medium (Ham) (+L-glutamine) with 10 % FBS and 1 % L-glutamine (Invitrogen; made according to manufacturers instructions). F12 medium is used with AGS cells, and was included for reference with the invasion assays in Chapter 3.

Bacteria were grown overnight to an OD_{650} of 0.10 to 0.15, and approximately $1x10^7$ bacteria/ml were added to 4.5 ml of each medium. The broth cultures were incubated for 4 hrs at 37˚C in 10 % CO_2 with constant rotation and growth was determined by a change in absorbance over this time. Bacteria were recovered from the medium by centrifugation (5 min at 14,000 rpm) and washed twice in phosphate buffered saline (PBS). Bacterial pellets were stored at –20˚C prior to use.

Lysates were prepared by resuspending the washed bacterial pellets in 1 ml PBS and sonicating on an OmniRupter 4000 sonicator (Omni International) for four, 30 sec intervals. The lysates were then centrifuged (5 min, 14,000 rpm) to remove cellular debris. The concentration of protein in the supernatant (lysate) was determined using a modified Lowry procedure (Markwell *et al.* 1998) with bovine serum albumin (BSA) used to generate a standard curve.

The samples were run on 12.5 % acrylamide gels and visualized using a dual silver stain (see Appendix I; Keenan *et al.* 1997). Approximately 2 μg of protein from each condition, with an equal amount of reducing buffer, was heated at 99°C for 5 mins before being loaded onto the gel. A prestained protein ladder (Fermentas) was used as a molecular weight marker. Gels were run for approximately an hr at 250 volts, 40 milli-amps and 15 watts. The gel was fixed overnight in a solution of 50 % ethanol and 10 % acetic acid in dH_20. The fixative was then removed, and replaced with 5 % ethanol and 1 % acetic acid in dH_20 and gently agitated for 15 mins. Periodic acid (0.7 %) was added during the last 10 mins. This alters carbohydrate moieties so that they stain with silver and thus

helps visualize bacterial LPS O chains (Keenan *et al*. 1997). Gels were washed in dH_20 (three 10 min washes) before staining with 0.1 % silver nitrate for 30 min. After a brief dH_20 wash, the gel was developed in 3 % sodium carbonate with 0.05 % formaldehyde until banding became apparent. Development was stopped with 1 % acetic acid. Where necessary, the gels were washed in Farmers Reducer (see Appendix I; Heukoshoven and Dernick 1985) for 10-30 sec to reduce background staining before being restained (as above). The gel was imaged with a FluorS Mutilmager imager (BioRad).

2.3 Results

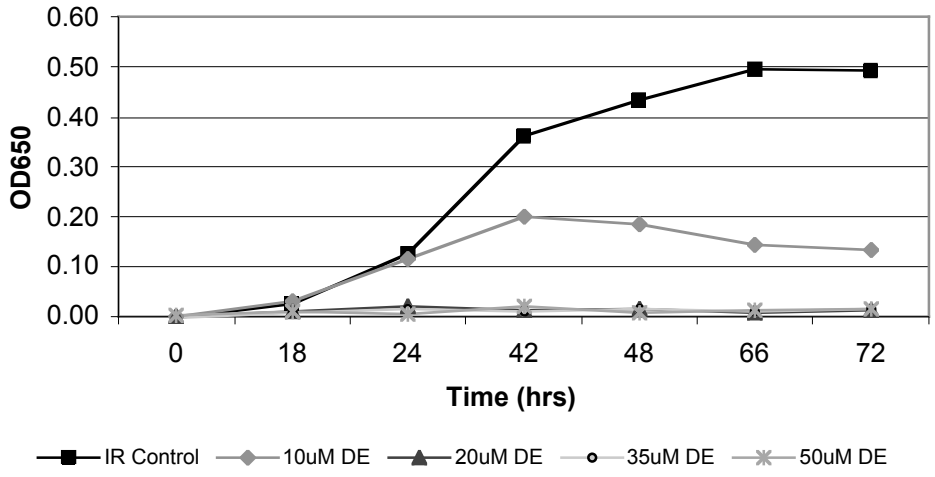
2.3.1 Determining the Minimal Inhibitory Concentration of DE

H. pylori was grown in iron-replete medium supplemented with varying concentrations of the iron-chelator DE to find the minimal inhibitory concentration (MIC) of iron chelator. Growth was measured as a function of optical density at 650 nm, and compared against the iron-replete positive control.

Bacteria ceased to grow in most conditions after 72 hrs, occasionally forming aggregates at the bottom of wells that sharply increased optical density. As such, overall levels of growth was compared between conditions at 48 hrs. Optical density measures total biomass, and it is possible that growth rate is simply being reduced in some conditions, such that growth levels could reach the iron-replete control in time. Yet many conditions reached stationary phase without achieving the same levels of growth as the iron-replete control, suggesting the partial iron-limitation is not just slowing growth rate but reducing total biomass of the bacteria.

H. pylori strain 60190 was used as a standard. Two other strains (SS1 and Tx30a) were then tested to see if they exhibited similar patterns to strain 60190. Over a 72 hr period, DE was capable of slowing (10 μM DE) or preventing ($\geq$ 20 μM) growth of strain 60190 (Figure 2.1).

A.



B.

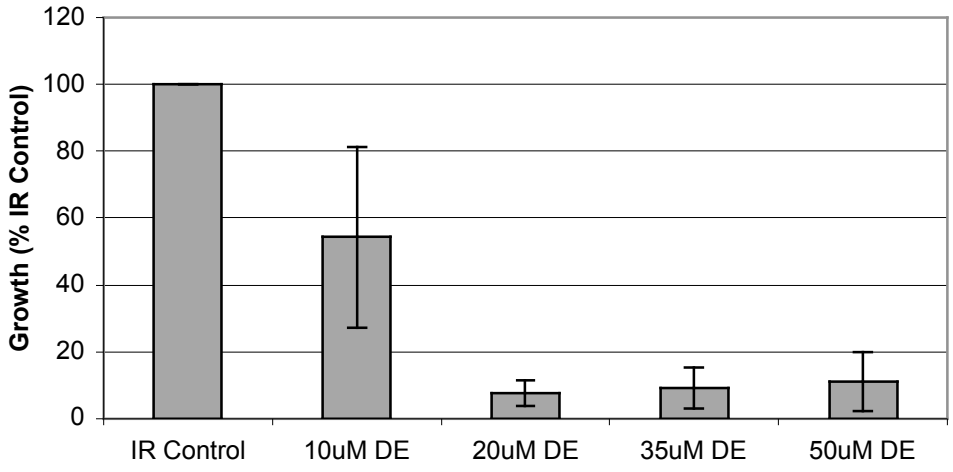
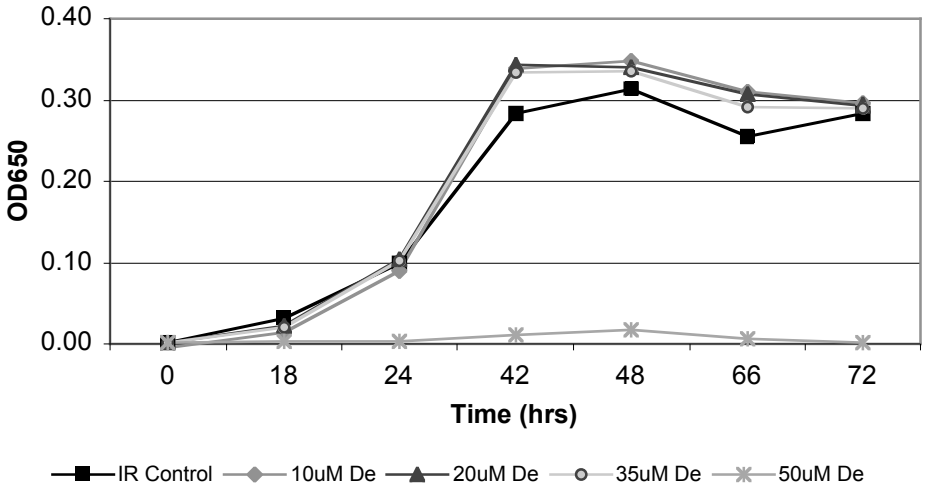


Figure 2.1: Effect of increasing iron-limitation on the growth of *H. pylori* strain 60190. Bacteria were grown in iron-replete medium (IR Control) with increasing concentrations (10-50 μM) of iron-chelator DE. (A) Growth over 72 hrs from one representative experiment. (B) Growth at 48 hrs relative to the positive control, mean of three independent experiments ±SE.

Strain 60190 was also grown in this same series of concentrations of DE with the addition of 10 μM $FeCl_3$ to determine if growth could be recovered with the addition of iron. Growth was recovered at concentrations of ≤ 35 μM DE (Figure 2.2).

A.



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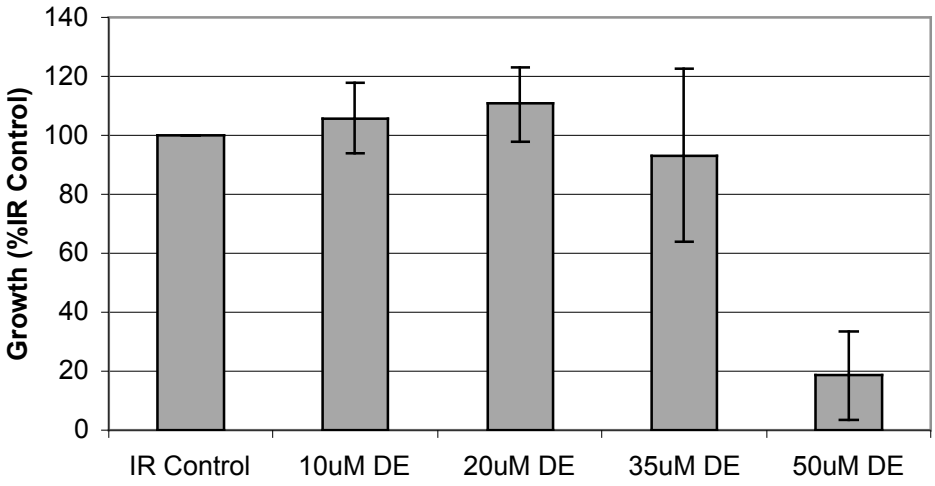


Figure 2.2: Iron supplementation rescues growth of *H. pylori* strain 60190 in iron-limiting conditions. Bacteria were grown in iron-replete medium (IR control) and iron-limited medium (with10-50 μM DE) supplemented with 10 μM $FeCl_3$ to determine if it was sufficient iron for recovery of growth. (A) Growth over 72 hrs from one representative experiment. (B) Growth at 48 hrs relative to the positive control, mean of three independent experiments ±SE.

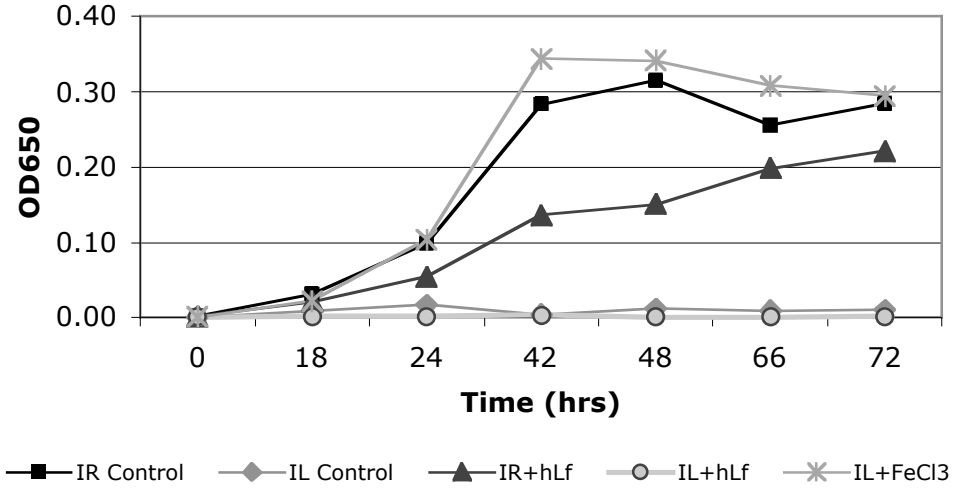
Strains SS1 and Tx30a were grown in medium supplemented with 10-50 μM DE, with and without 10 μM $FeCl_3$. Each experiment was performed once to determine if a similar pattern of growth occurred across strains (see Appendix II). At least partial growth inhibition was seen in all strains with 20 μM DE and the addition of 10 μM $FeCl_3$ reversed the effect. From this, iron-limited medium was defined as BB + 5 % FBS supplemented with 20 μM DE, and used for all further growth assays.

2.3.2 Growth of H. pylori with 0.5 mg/ml of Partially Iron-Saturated Human Lactoferrin

To see if hLf affected the growth of *H. pylori*, strains were grown in both iron-replete and iron-limited media supplemented with hLf. Growth of all *H. pylori* strains was measured in medium supplemented with 0.5 mg/ml of Lf from human milk, about 25 % iron-saturated. This is equivalent to approximately 3 μM iron in solution.

Similar to previous results, growth in strain 60190 was significantly reduced with the additional of 20 μM DE (p-value <0.001) and recovered with the addition of $FeCl_3$ (p-value <0.001). The addition of 0.5 mg/ml hLf to iron-replete and iron-limited conditions did not significantly change the growth of strain 60190, although small decreases in absorbance when compared to the iron-replete or iron-limited control were apparent, suggesting some growth inhibition (Figure 2.3).

A.



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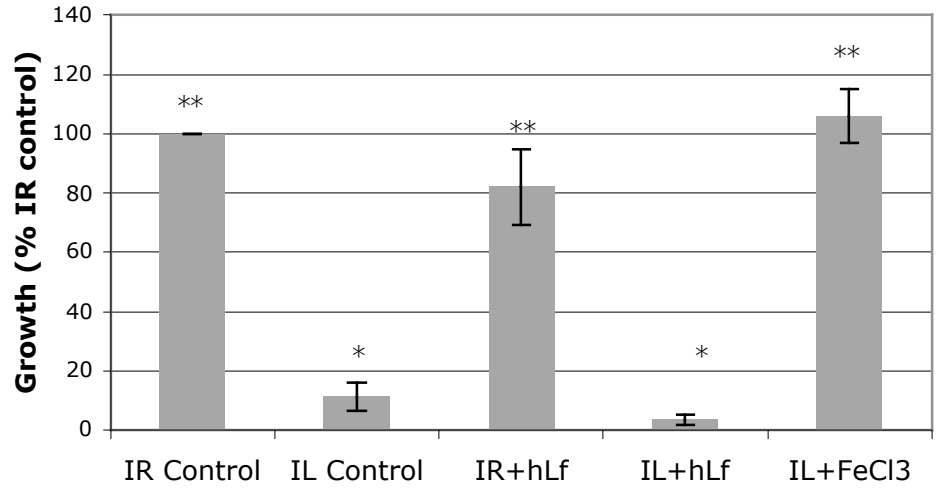
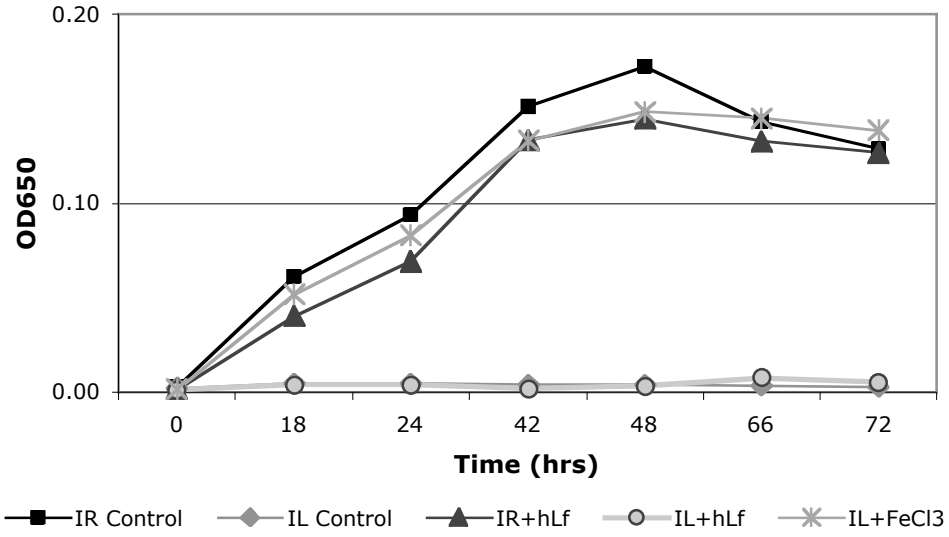


Figure 2.3: Growth of strain 60190 with 0.5 mg/ml of partially iron-saturated human lactoferrin. Bacteria were cultured in iron-replete (IR control) and iron-limited (with 20 μM DE; IL control) media. Media were supplemented with ~25 % iron-saturated hLf or 10 μM $FeCl_3$. (A) Growth over 72 hrs from one representative experiment. (B) Growth of 48 hrs relative to the positive control, mean of three independent experiments ± SE. *, results are significantly different than iron-replete control. **, results are significantly different than the iron-limited control ($p < 0.05$ by Student T-test).

Overall, strain SS1 grew poorly, only reaching to an OD_{650} of 0.20, but growth relative to the control was similar across conditions to that observed for strain 60190 (Figure 2.4). Again, growth was significantly reduced with the additional of 20 μM DE (p-value <0.001) and recovered with the addition of $FeCl_3$ (p-value <0.001). The addition of 0.5 mg/ml of partially iron-saturated hLf to iron-replete conditions significantly reduced total growth levels of strain SS1, to about 80 % of the control at 48 hrs (p-value <0.001; Figure 2.4). The addition of 0.5 mg/ml hLf to iron-limiting conditions did not significantly reduce growth, though a small decrease in total growth level was apparent.

A.



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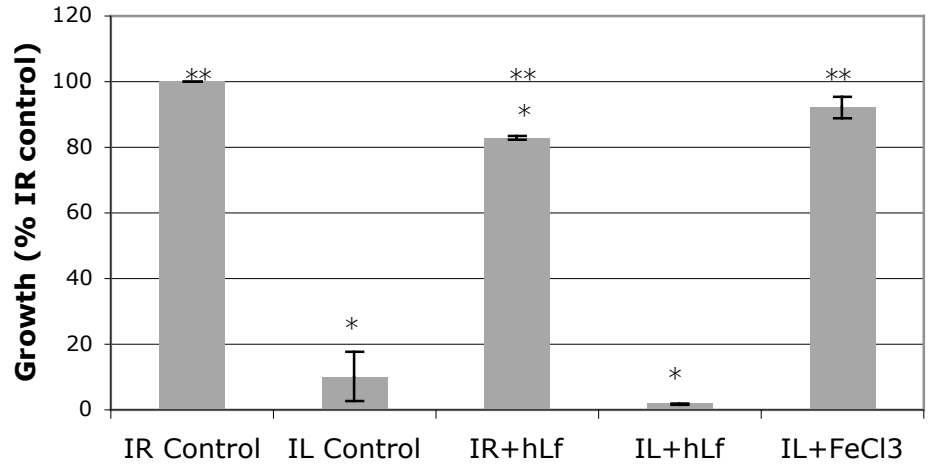


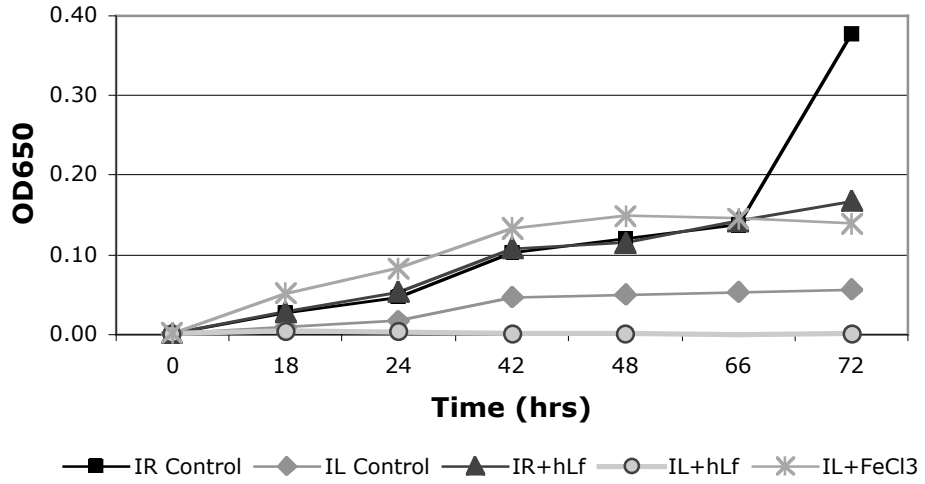
Figure 2.4: Growth of strain SS1 with 0.5 mg/ml of partially iron-saturated human lactoferrin, with growth inhibition in iron-replete conditions. Bacteria were grown in iron-replete (IR control) and iron-limited (with 20 μM DE; IL control) media. Media were supplemented with ~25 % iron-saturated hLf or 10 μM $FeCl_3$. (A) Growth over 72 hrs from one representative experiment. (B) Growth at 48 hrs relative to the positive control, mean of three independent experiments ± SE. *, results are significantly different than iron-replete control. **, results are significantly different than the iron-limited control ($p < 0.05$ by Student T-test).

Growth of strain Tx30a was only partially inhibited in medium with 20 μM DE, but was still significantly lower than the iron-replete control (p-value <0.001). Growth was recovered in iron-limited medium with 10 μM $FeCl_3$, but not up to the level of the iron-replete control (84 %). The large increase in growth in the iron-replete control at 72 hrs was due to clumping of non-viable bacteria at the bottom of each well.

No affect was seen with the addition of 0.5 mg/ml of partially iron-saturated hLf to iron-replete medium. The addition of hLf to iron-limiting conditions significantly reduced the growth level achieved by strain Tx30a by over 70 % (p-value <0.001; Figure 2.5). This may represent a strain-specific difference in iron uptake or intracellular iron stores, as has been reported elsewhere (Bland *et al*. 2004). Alternatively, the effect of adding hLf may not have been as notable in strains 60190 and SS1 as growth in iron-limiting conditions was already quite low.

From this, there is no evidence that any of these three strains of *H. pylori* are using partially iron-saturated hLf as an iron-source. The presence of 0.5 mg/ml of hLf decreased growth levels of strains 60190 and SS1 by about 20 %, although it was only significant for the latter (Table 2.1). Growth levels of all strains was reduced in iron-limited media supplemented with hLf. This reduction could be due to an hLf chelation of iron in the media.

A.



B.

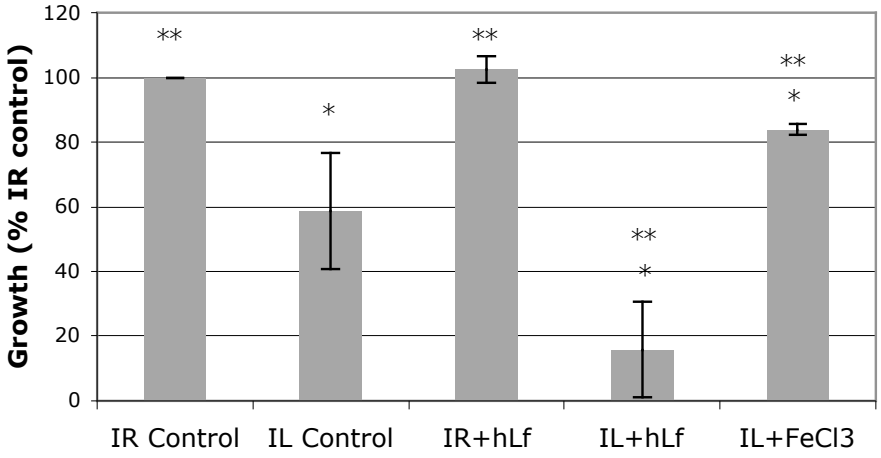


Figure 2.5: Growth of strain Tx30a with 0.5 mg/ml of partially iron-saturated human lactoferrin, with growth inhibition in iron-limiting conditions. Bacteria were grown in iron-replete (IR control) and iron-limited (with 20 μM DE; IL control) media. Media were supplemented with ~25 % iron-saturated hLf or 10 μM $FeCl_3$. (A) Growth over 72 hrs from one representative experiment. (B) Growth at 48 hrs relative to the positive control, mean of three independent experiments ± SE. *, results are significantly different than iron-replete control. **, results are significantly different than the iron-limited control ($p < 0.05$ by Student T-test).

Table 2.1: The effect of 0.5 mg/ml of partially iron-saturated human lactoferrin on *H. pylori* growth[1]

Strain	IR medium[2]	IL medium[2]	IR+hLF	IL+hLF	IL+$FeCl_3$[3]
60190	100	11 ± 4.7	82 ± 13	3.5 ± 1.7	106 ± 9.3
SS1	100	10 ± 7.5	82 ± 0.66	1.6 ± 0.33	92 ± 3.2
Tx30a	100	58 ± 18	102 ± 4.1	16 ± 15	84 ± 1.6

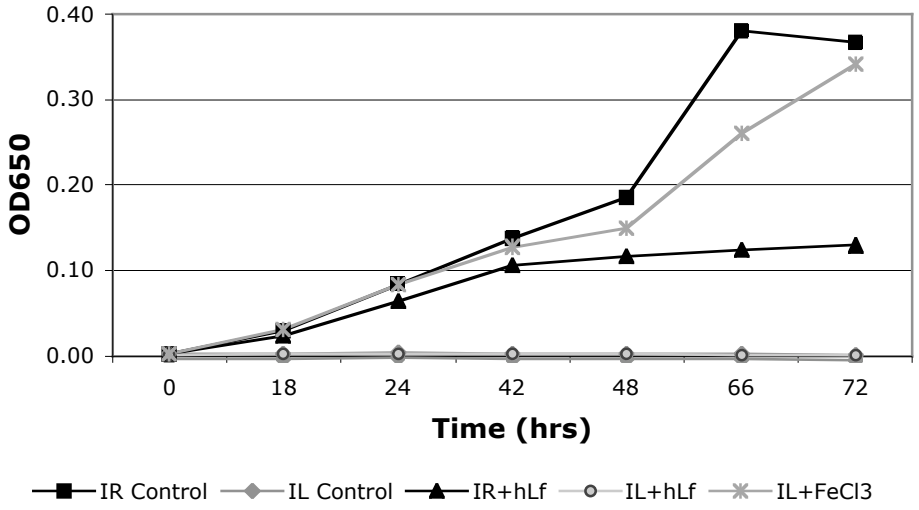
(1) Growth expressed as percentage of the iron-replete control (IR) over three experiments, ±SE.
(2) Iron-replete medium is defined as BB with 5 % FBS. Iron-limited medium (IL) is BB with 5 %FBS and 20 μM DE.
(3) Iron-limited medium was supplemented with 10 μM $FeCl_3$ to determine if growth could be recovered with the addition of iron to the medium.

2.3.3 Growth of H. pylori strain 60190 with 1.0 mg/ml of Partially Iron-Saturated Human Lactoferrin

To see if the addition of more hLf might have a measurable effect on growth, *H. pylori* strain 60190 was cultured with 1.0 mg/ml of partially iron-saturated hLf. This concentration of hLf potentially increased both the possible inhibitory effect of hLf, and the amount of iron (~6 μM iron) that would be available if *H. pylori* were able to use hLf as an iron source.

As observed previously with strain 60190, growth was significantly reduced with the addition of 20μM DE to the medium, and growth was recovered with the addition of $FeCl_3$ (Figure 2.6). There was no significant difference between growth in iron-limited medium with the addition of 1 mg/ml hLf. If partially iron-saturated hLf is able to chelate remaining free iron in the medium, increasing the concentration of hLf should result in an equal or stronger inhibition of growth than seen with 0.5 mg/ml. However, growth in iron-limited medium was quite low, only 1% of the iron-replete positive control, making additional inhibition from hLf difficult to detect. Nevertheless, growth on iron-replete conditions was decreased by 30 % with 1.0 mg/ml of partially iron-saturated hLf (Table 2.2).

A.



B.

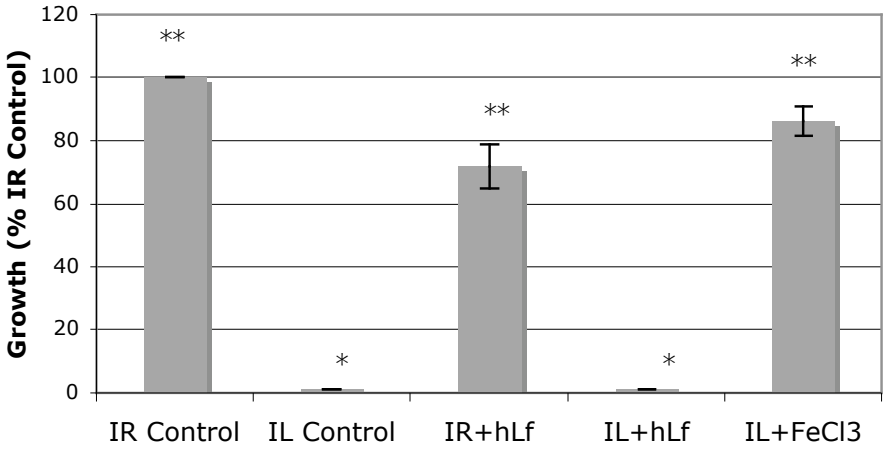


Figure 2.6: Possible growth inhibition of strain 60190 with 1 mg/ml of partially iron-saturated human lactoferrin. Bacteria were grown in iron-replete (IR control) and iron-limited (with 20 μM DE; IL control) media. Media were supplemented with ~25 % iron-saturated hLf or 10 μM $FeCl_3$. (A) Growth over 72 hrs from one representative experiment. (B) Growth at 48 hrs relative to the positive control, mean of three independent experiments ± SE. *, results are significantly different than iron-replete control. **, results are significantly different than the iron-limited control ($p < 0.05$ by Student T-test).

Table 2.2: The effect of 1 mg/ml of partially iron-saturated human lactoferrin on *H. pylori* growth[1]

Strain	IR medium[2]	IL medium[2]	IR+hLF	IL+hLF	IL+$FeCl_3$[3]
60190	100	1 ± 0.017	72 ± 6.9	1 ± 0.058	86 ± 4.8

(1) Growth expressed as percentage of the iron-replete control (IR) over three experiments, ±SE.
(2) Iron-replete medium is defined as BB with 5 % FBS. Iron-limited medium (IL) is BB with 5 %FBS and 20 μM DE.
(3) Iron-limited medium was supplemented with 10 μM $FeCl_3$ to determine if growth could be recovered with the addition of iron to the medium.

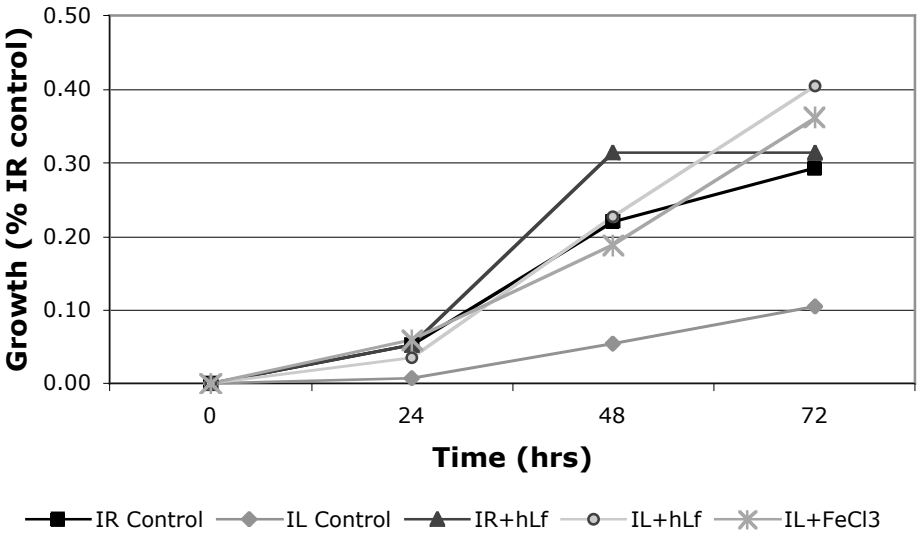
2.3.4 Growth of H. pylori strain 60190 with 0.5 mg/ml of Iron-Saturated Human Lactoferrin

To see if the level of hLf iron saturation affects the ability of *H. pylori* to use hLf, the growth of strain 60190 was tested with 0.5 mg/ml of iron-saturated (holo) hLf (~85 % iron-saturated). This brought the total Lf-bound iron to 10 μM, the same as used in the $FeCl_3$ controls.

Growth of strain 60190 was only partially reduced with the addition of 20 μM DE to the medium, but the reduction was still significant (p-value <0.001). Growth was recovered with the addition of 10 μM $FeCl_3$ (Figure 2.6).

Addition of holo-hLf to iron-limited medium resulted in significantly increased growth when compared to the iron-limited control (p-value<0.05). In iron-replete medium, the addition of holo-hLf resulted in a substantial increase in growth (p-value 0.062) (Table 2.3).

A.



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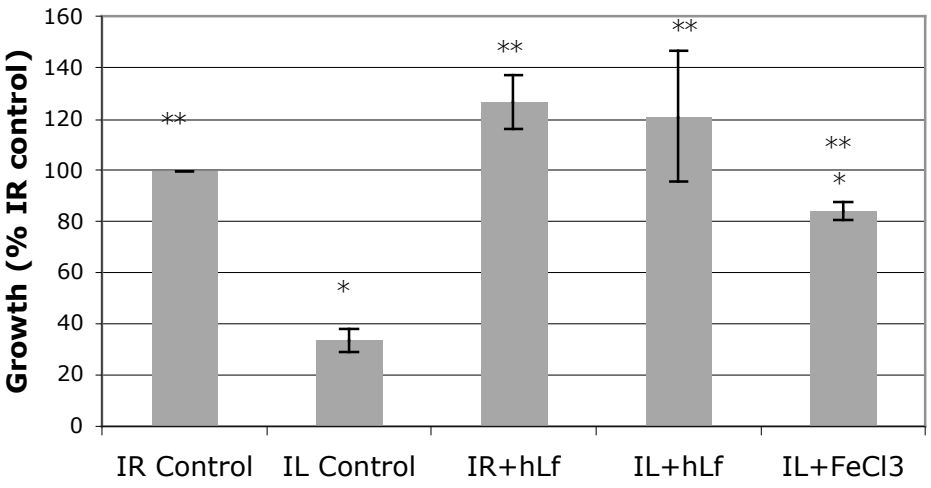

Figure 2.7: Growth recovery of strain 60190 in iron-limiting conditions with 0.5 mg/ml of fully iron-saturated human lactoferrin. Bacteria were grown in iron-replete (IR control) and iron-limited (IL control) media (with 20 μM DE; IL control). Media were supplemented with ~85 % iron-saturated hLf or 10 μM $FeCl_3$. (A) Growth over 72 hrs from one representative experiment. (B) Growth at 48 hrs relative to the positive control, mean of three independent experiments ± SE. *, results are significantly different than iron-replete control. **, results are significantly different than the iron-limited control ($p < 0.05$ by Student T-test).

Table 2.3: The effect of 0.5 mg/ml of fully iron-saturated human lactoferrin on *H. pylori* growth[1]

Strain	IR medium[2]	IL medium[2]	IR+hLF	IL+hLF	IL+$FeCl_3$[3]
60190	100	34 ± 0.030	130 ± 4.7	120 ± 10	84 ± 3.5

(1) Growth expressed as percentage of the iron-replete control (IR) over three experiments, ±SE.
(2) Iron-replete medium is defined as BB with 5 % FBS. Iron-limited medium (IL) is BB with 5 %FBS and 20 μM DE.
(3) Iron-limited medium was supplemented with 10 μM $FeCl_3$ to determine if growth could be recovered with the addition of iron to the medium.

2.3.5 Phenotypic Changes in H. pylori in Iron-limited Conditions

The ability of iron-limitation to induce changes in the phenotype of *H. pylori* strain 60190 was examined. Bacteria were grown in iron-replete and iron-limited media BB + 5 % FBS and F12 nutrient broth (+ 10 % FBS) for 4 hrs. Growth was measured at OD_{650} before and after incubation for all conditions to see if there was a difference in growth between conditions (Table 2.4). The bacteria were lysed, and lysates were examined using SDS-PAGE and silver staining. Whereas there was little evidence of growth inhibition, differences in bacterial phenotype were observed after only 4 hrs in iron-limiting media.

Table 2.4: Growth of *H. pylori* over four hours in iron-replete and iron-limited conditions

	IR-BB[1]	IL-BB[1]	IR-F12[1]	IL-F12[1]
T0	0.059	0.060	0.060	0.053
T4	0.075	0.072	0.062	0.059

(1) Growth expressed as OD_{650} in iron replete (IR) and iron-limited (IL) BB + 5 % FBS and F12 nutrient medium (+10 % FBS) at the start (T0) and after four hrs incubation (T4).

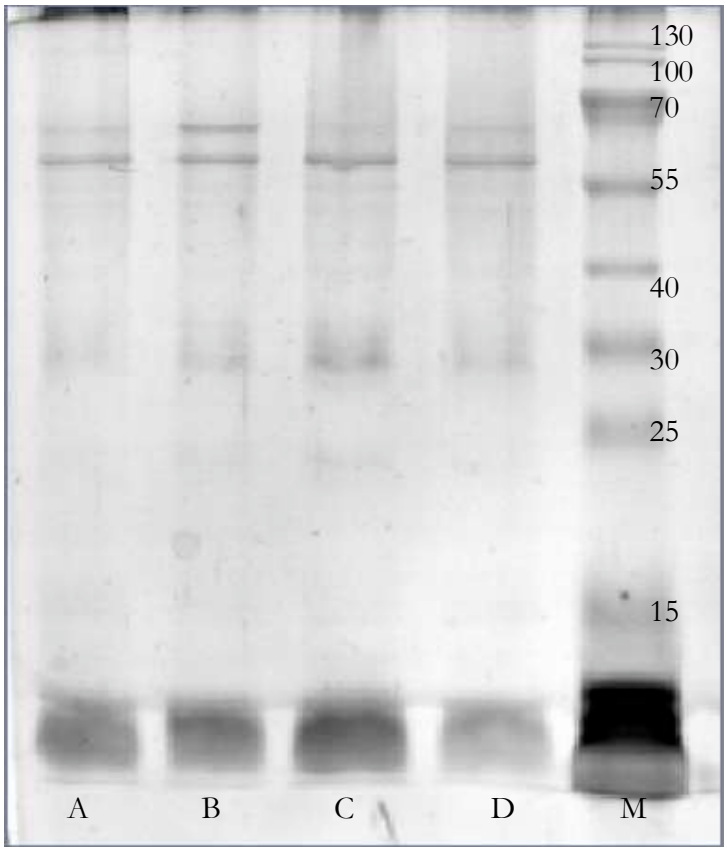

Figure 2.8: Phenotypic changes in HP60190 grown in iron-limited conditions. Bacteria were grown for 4 hrs in iron-replete and iron-limited media (with 20μM DE). (A) iron-replete BB (B) iron limited BB (C) iron-replete F12 nutrient medium (D) iron-limited F12 nutrient medium (M) prestained protein ladder, in kDa. Bacterial lysates were visualized on 12.5 % acrylamide gel by SDS-PAGE and silver stained.

There is an apparent increase in protein, approximately 70 kDa, in the iron-limiting-BB conditions. There is an analogous, though less evident, increase in the same band in the IL-F12 nutrient medium. Comparing inter-lane staining, it appears that lane (D) may have been load with slightly less protein (C), meaning that the increased density of the staining of the 70 kDa band could be even higher than it first appears in iron-limiting-F12 medium. These results suggest that even in the 4 hr time

limit of the assay, some changes in bacterial phenotype are evident in iron-limiting media.

2.4 Discussion

Lactoferrin is an iron-binding protein, chelating free iron where it is produced on mucosal surfaces in mammals. Human Lf on the mucosal layer of the stomach is known to increase during *H. pylori* infection (Choe *et al.* 2003, Wen *et al.* 2004). In the tens of thousands of years that *H. pylori* has infected humans (Blaser 1998, Falush *et al.* 2003), it is likely that this bacterium has developed some adaptations to living in a high Lf environment. Indeed, despite the presence of the usually bacteriostatic Lf, *H. pylori* infections are highly persistent, rarely being cleared by the host without additional therapeutics (Lee *et al.* 1993, Kusters *et al.* 2006). Previous studies have suggested that *H. pylori* may be able to use iron bound to hLf for growth, but the data is unclear and the possible mechanism has yet to be determined.

To test the ability of *H. pylori* to use hLf, bacteria were grown in iron-replete and iron-limited media supplemented with hLf. First, the minimal concentration of the iron-chelator DE capable of inhibiting growth of strains 60190, SS1, and Tx30a was determined. It was found that 20 μM DE was sufficient to completely prevent growth in 60190 and SS1 and partially inhibit growth in Tx30a. From this, iron-limited medium was defined as the addition of 20 μM DE to iron-replete Brucella broth. In all cases, the addition of 10 μM $FeCl_3$ to iron-limited medium resulted in growth similar to the positive iron-replete control, suggesting the iron-limitation created by DE can be overcome by supplementation with additional iron sources.

2.4.1 Affect of Partially Iron-Saturated Human Lactoferrin on the Growth of H. pylori

Growth of *H. pylori* was tested in iron-replete and iron-limiting conditions with 0.5 and 1 mg/ml of partially iron-saturated hLf. The

addition of partially iron-saturated hLf to iron-replete conditions led to a reduction of growth in strains 60190 and SS1, but not Tx30a. A 20 % decrease in the level of growth at 48 hrs was apparent with the addition of 0.5 mg/ml of hLf in strains 60190 and SS1, though only significant in the latter. When the amount of hLf was increased to 1 mg/ml, growth of strain 60190 was reduced by 30 % in iron-replete conditions.

Growth of all three strains was decreased in iron-limited medium supplemented with partially iron-saturated hLf, possibly due to chelation of remaining iron in the medium. This decrease was only significant with strain Tx30a. Growth in iron-limited conditions was already low in strains 60190 and SS1, making it difficult to detect further inhibition with the addition of hLf. A similar issue arose with the addition of 1 mg/ml of partially iron-saturated hLf, where growth in iron-limiting conditions was as low as 1 % of the control. Otherwise, were the decrease in growth due to iron-chelation, additional hLf would be expected to exert an even larger degree of inhibition.

Partially iron-saturated hLf appears to decrease growth in both iron-replete and iron-limited conditions. This could be due to hLf chelating iron in the medium. Human Lf has a range of antimicrobial activities, and it is possible that hLf is affecting growth through a mechanism unrelated to iron-content. This is unlikely, though, in light of the increase in growth with fully iron-saturated hLf discussed below.

2.4.2 Affect of Fully Iron-Saturated Human Lactoferrin on the Growth of H. pylori

The level of iron-saturation of hLf can affect its ability to chelate additional iron from the medium, as well as increase the amount of Lf-bound iron available for use. Whereas partially iron-saturated hLf decreased growth of strain 60190 in iron-replete medium, the addition of 0.5 mg/ml of holo-hLf led to a 30 % increase in growth. This effect was

notably increased in iron-limited medium, bringing growth up to the level of the iron-replete control.

However, partially and fully iron-saturated hLf could be affecting growth differently because of discrepancies in the total amount of Lf-bound iron being added to the medium. Only 3 - 6 μM of iron is present in 0.5-1.0 mg/ml of partially iron-saturated hLf, whereas 0.5 mg/ml of holo-hLf translates to approximately 10 μM of iron under these culturing volumes. Iron-replete medium (Brucella broth with 5 % FBS) would be expected to have 1.63 – 2.94 μM free iron (Worst *et al*. 1995). If *H. pylori* were able to access all Lf-bound iron, both partially and fully iron-saturated Lf should be sufficient for growth. However, the efficiency at which the bacteria are able to utilize the various forms of iron probably differs.

One preliminary trial was performed with 0.25 mg/ml of holo-hLf, representing approximately 5 μM of iron in solution, and growth recovery in iron-limiting conditions was not evident. It is possible then, that the quantity of Lf-bound iron was insufficient for growth with the partially iron-saturated hLf.

Iron deficiency does not, however, account for the decrease in growth seen with partially iron-saturated hLf. It may be that iron bound to holo-hLf is more accessible to *H. pylori*. Human serum, with 30 % iron-saturated transferrin, is usually inhibitory to the growth of *Candida albicans*, yet the fungus grows profusely in serum with 100 % saturated transferrin (Bullen 2005). Furthermore, the conformation of Lf changes as it binds more iron, which could affect how it interacts with receptors. Holo-Lf has a closed structure, with iron release dependent on destabilization of the closed form either by receptor binding or a low pH (Mazurier and Spik 1980, Baker 2005). Whether or not levels of iron-saturation affect the ability of *H. pylori* to use hLf would depend on the mechanism by which it is obtaining the iron.

2.4.3 Potential Mechanisms of Acquiring Iron from Human Lactoferrin

The mechanism by which *H. pylori* is able to access iron bound to hLf is unknown. *H. pylori* is known to express a number of iron-regulated outer membrane proteins (IROMPS) (Lee *et al.* 2009), some of which are associated with uptake of iron from host iron-binding proteins (Dhaenens *et al.* 1997, Worst *et al.* 1995). Changes in the *H. pylori* phenotype was observed within 4 hrs of iron-limitation, and some of these may be proteins involved in the uptake of iron from hLf.

A number of other species have been shown to use Lf as an iron-source, often with membrane bound, tonB-like lactoferrin binding proteins. Expression of a lactoferrin binding protein has been suggested for *H. pylori* as well (Dhaenens *et al.* 1997), but the gene for the putative *H. pylori* LBP has not been identified.

H. pylori may also be able to acquire iron from Lf indirectly, without the need of an LBP. *H. pylori* has recently been described as having a riboflavin-mediated system of iron acquisition (Worst *et al.* 1998). Flavins are capable of reducing Fe(III) in iron-containing complexes to Fe(II) (Worst *et al.* 1998, Andrews *et al.* 2003). The more soluble Fe(II) can then diffuse into the cell through porin channels in the membrane. Interestingly, a similar system was described in *Listeria monocytogenes,* with a membrane-bound flavin reducatase increasing the accessibility of free iron from several iron-binding proteins, including siderophores, heme, transferrin, and lactoferrin (Deneer *et al.* 1995, Worst *et al.* 1998).

2.4.4 How Lactoferrin may be Affecting Growth in vivo

All strains tested here showed a decrease in growth with partially iron-saturated hLf and strain 60190 showed an increase in growth with

fully iron-saturated hLf. Most hLf in the body is not fully iron-saturated (Bullen 2005) and the partially iron-saturated hLf used in this study, which was isolated from human milk, has a similar level of iron-saturation as hLf *in vivo*. However, iron levels in the stomach are subject to large variations (van Vliet *et al.* 2002) and saturation of Lf in the stomach could be changing as well. Differing degrees of iron-saturation may possibly account for diverging reports on whether or not Lf is an affective therapeutic for *H. pylori in vivo*.

It is interesting to note that the Miehlke *et al.* study showing rhLf reduced *H. pylori* growth used rhLf that was 95 % iron-free (Miehlke *et al.* 1996). As mentioned previously, rhLf may affect *H. pylori* differently than hLf from human milk due to differing post-translational modifications, as has been suggested elsewhere with *Shigella* spp. (Gomez *et al.* 2003). It may be, though, that iron-free Lf is able to exert a stronger effect against bacterial growth, acting as larger sink for iron than similar amounts of partially iron-saturated hLf isolated from human milk.

It would be interesting to see if the increase in growth seen with fully iron-saturated hLf in strain 60190 was also seen with strains SS1 and Tx30a. Various studies showing an increase in *H. pylori* growth used either partially or fully iron-saturated hLf, though with strains other than used here (Husson *et al.* 1993, Velayudhan *et al.* 2000). Strain-specific differences in *H. pylori*'s ability to use hLf could contribute to its persistence in the host and possibly even virulence. Some patients with *H. pylori*-related, iron-deficient anemia are colonized with strains that produce more IROMPs and are more efficient users of iron (Lee *et al.* 2009).

Chapter 3

The Effect of Lactoferrin on the Frequency of Internalization of *Helicobacter pylori* into Human Epithelial Cells

3.1 Experimental Justification

Lactoferrin, particularly bovine Lf (bLf), has been shown to reduce the invasion of epithelial cells by a number of pathogens (Ajello *et al.* 2002, Di Biase *et al.* 2004, Superti *et al.* 2005). To date, however, little is known about how Lf affects internalization in species that are adapted to high levels of human Lf (hLf) and potentially express Lf binding proteins (LBP).

Previous work suggests that *H. pylori* has a low rate of internalization into epithelial cells (Peterson and Krogfelt 2003), which may be affected by interacting with hLf. Human Lf may decrease internalization of *H. pylori* into human epithelial cells, as has been shown with other species, or have no effect, as is possible if *H. pylori* adapts to LF in the stomach. It is also possible that hLf could increase the invasiveness of *H. pylori*. This hypothesis is supported by evidence of a putative *H. pylori* LBP that has been described as hLf-specific (Dhaenens *et al.* 1997). By directly binding hLf that is bound to the epithelial cell surface (Legrand *et al.* 2004), *H. pylori* may be passively internalized. This effect may be different from that of bLf, which binds bacterial and cellular surfaces (Valenti *et al.* 2005) but does not have a high affinity for hLf-specific receptors (Dhaenens *et al.* 1997).

The commonly used gentamycin protection assay was used to enumerate internalized *H. pylori*, and to then derive a frequency of

internalization. In this assay, gastric adenocarcinoma (AGS) cells were infected with bacteria for 4 hrs, washed, and then incubated for two hrs with gentamycin to kill any extracellular bacteria. Cells were lysed and any bacteria recovered from the lysate were presumed to have been intracellular because they were in this way protected from exposure to gentamycin. The internalization assay was performed under both iron-replete (as a positive control) and iron-limiting conditions. Iron-limited medium was created by two methods: firstly by removing fetal bovine serum (FBS), which contains iron, and secondly by adding the iron-chelator desferoxamine mesylate (DE) to FBS-supplemented F12 medium.

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3.2 Materials and Methods

3.2.1 Bacterial Strains and Growth Conditions

Bacteria were grown and maintained as described in Chapter 2 (refer to page 31). All assays were done with *H. pylori* strain 60190.

3.2.2 Cell culture

AGS cells (ATCC CRL-1739) were grown at 37˚C in F12 nutrient medium. Antibiotics and FBS were omitted to generate F12 nutrient medium without antibiotics or serum respectively. For assays, AGS cells were grown in 24 well plates (BD), seeded with 1x 10^5 cells per well and grown overnight to approximately 2 x 10^5 cells.

3.2.3. Determining Efficacy of Gentamycin

To determine if incubation with gentamycin can kill *H. pylori*, 100 μg/ml of gentamycin (Gibco) was added to F12 nutrient medium without antibiotics. Overnight cultures of bacteria were added to the medium and incubated for two hrs at 37˚C in 10 % CO_2 under constant rotation (120 rpm). Samples of 200 μl were taken at 30 min intervals and plated on Columbia blood agar plates. The plates were incubated at 37˚C in 10 % CO_2 and *H. pylori* colony-forming units (CFUs) were quantified four days later.

3.2.4 Determining Cell and Bacterial Viability in Medium Without FBS

The effect of medium without FBS on AGS cells and *H. pylori* viability during the 4 hr period of the gentamycin protection assay was determined. AGS cells (2 x 10^5) in 24 well plates were incubated with F12

medium with and without the addition of 10 % FBS for 4 hrs at 37˚C. AGS cells were examined under light microscopy for morphological signs of stress. Separately, *H. pylori* was recovered from an overnight broth by centrifugation (10 min at 10,000 rpm), resuspended in F12 medium (with and without FBS) and incubated for 4 hrs at 37˚C in 10 % CO_2 with rotation. The number of *H. pylori* in each condition was quantified via serial dilutions on Colombia blood agar plates.

3.2.5 Determining Efficacy of Lysis Buffers

An assay was performed to determine if potential lysis buffers were able to efficiently lyse AGS cells. AGS cells (2×10^5), grown overnight in a 24 well plate, were washed 3 times in PBS before the addition of 1 ml of lysis buffer that included F12 medium without antibiotics (control), 0.5 % saponin in PBS, 0.05 % saponin in PBS, 0.25 % sodium deoxycholate (NaDoc; Difco) in H_20 (w/v), 1 % Nonidet P40 (BDH Chemicals LTD; see Appendix I) or H_20 adjusted to pH 11 with NaOH. Each well was aspirated five times with a pipette. Cells were examined for lysis at 5 min intervals.

3.2.6 Lysis Buffer Viability Assay

An assay was performed to determine if *H. pylori* could survive exposure to these lysis buffers. *H. pylori* were grown overnight in broth culture and 200 µl aliquots were centrifuged (10 min at 10,000 rpm) to recover bacteria. Each bacterial pellet was resuspended in 200 µl of lysis buffer (see above) and left to sit at room temperature, with 10 µl samples removed and cultured at 5, 10, and 20 min intervals. A more precise

quantification of bacterial viability was determined after 20 min of incubation via serial dilutions on Colombia blood agar plates.

3.2.7 Gentamycin Protection Assay

A gentamycin protection assay was performed to determine if, and at what frequency, *H. pylori* invades AGS cells. To prepare the inoculum for infecting the cultured cells, *H. pylori* were grown in broth culture overnight to an OD_{650} between 0.10 and 0.15. Aliquots of 0.5 ml of broth culture were centrifuged (10 min at 10,000 rpm), and the bacterial pellets were resuspended in antibiotic-free F12 medium with and without the addition of 10 % FBS. The number of bacteria in the inoculum was quantified via serial dilutions on Columbia blood agar plates.

AGS cells grown overnight in 24 well plates (2×10^5) were washed with PBS (three 500 μl washes) to remove antibiotics before infection with bacteria. Cells were infected with 50-80 μl of *H. pylori* inoculum, which equated to approximately 5×10^6 bacteria, giving a multiplicity of infection (MOI) of 25:1. To determine the effect of Lf on internalization in FBS-free, iron-limiting conditions, duplicate wells were supplemented with 1mg/ml of partially iron-saturated hLf.

In a separate experiment, internalization was tested in iron-replete (F12 nutrient medium + 10 %FBS) and iron-limited medium (F12 nutrient medium + 10 % FBS + 20 μM DE). Media were similarly inoculated with bacteria and added to washed AGS cells. In this experiment, additional wells were supplemented with 1 mg/ml of partially iron-saturated hLf, denatured hLf, or partially iron-saturated bLf from bovine milk (Sigma). The denatured hLf was prepared by heating hLf at 100˚C for five min.

Experiments were carried out at 37ºC for 4 hrs, to allow internalization to occur. After infection, individual wells were washed with the media used during the infection step. The AGS cells were then incubated with 100 μg of gentamycin for 2 hrs, using the same media conditions, before being washed three times with F12 medium. The final wash, which was retained as a medium control, was supplemented with 100 μl of lysis buffer to show there were no remaining bacteria in solution or in non-adherent cells. One ml of 0.5 % saponin in PBS (lysis buffer) was added to each well and aspirated five times before being left to sit for 10 min. The wells were aspirated again before collection of the AGS cell lysate.

Bacteria in the lysate were quantified via serial dilutions on blood agar plates, and the plates were incubated for 4 days at 37˚C in 10 % CO_2. For each condition, overall internalization frequency was calculated as percent of the original inoculum recovered after incubation with gentamycin. For comparison across conditions, the number of intracellular bacteria was normalized to the iron-replete control for that experiment. Statistical significance between conditions was determined with a Student t-test.

3.2.8 Adhesion Assay

AGS cells were grown overnight and inoculated with bacteria as in the gentamycin protection assay, testing iron-limitation via DE. Infection was carried out at 37˚C for 4 hrs.

After infection, wells were washed three times with F12 medium. Thereafter, 1 ml of 0.5 % saponin in PBS was added to each well, and the wells were aspirated five times, allowed to sit 10 min, and aspirated again.

Bacteria in the lysis buffer were quantified via serial dilutions on blood agar plates, and the plates were incubated for 4 days at 37˚C in 10 % CO_2. Adhesion frequency was calculated as percent of the original inoculum recovered after infection. For comparison across conditions, the number of intracellular bacteria was compared to the iron-replete control. Statistical significance was determined between conditions with a Student t-test.

3.2.9 Analysis of the Statistical Power of the Internalization and Adhesion Assays

Statistical power is defined as the probability of rejecting the null hypothesis when it is false and should be rejected (Roush and Tozer 2003). If the difference between conditions are not statistically different, that could suggest the actual effect size between conditions is smaller than the measured effect size and more replicas are needed.

A power analysis was carried out using the free web program G*Power3 (Erdfelder 1996) to determine the number of additional replicas that would be needed to get statistically significant differences between conditions, taking into account the variation seen in the initial data. The measured effect size between two conditions was determined using the standard deviation and means from the initial data set for both the internalization and adhesion assay using DE-mediated iron-limitation. Effect size was calculated as below, where mu1 is the mean of population 1 and mu2 is the mean of population 2. Sigma is the standard deviation.

$$d = \frac{[\ mu1 - mu2\ ]}{sigma}$$

Because the sample sizes between conditions were the same, it is possible to standardize sigma from the two standard deviations as shown below, where sigma A2 is the standard deviation of population 1 and sigma B2 is the standard deviation of population 2:

$$\text{sigma}' = \sqrt{((\text{sigmaA2} + \text{sigmaB2})/2)}$$

The effect size and number of replicas was then entered into a post-hoc power analysis, which determines the power of a given set of data. For the internalization and adhesion assays, the calculated power was used in an a-prior power analysis to determine how many more replicas would be needed to detect other effect sizes at the $p<0.05$ level, given the degree of variation seen in the original data set.

3.3 Results

An initial set of experiments was performed to validate the conditions used during the gentamycin protection assay. Gentamycin was tested for its ability to kill *H. pylori*. The bacteria were incubated with 100 μg/ml of gentamycin for two hrs, with samples taken at T_o and thereafter at 30 min intervals. No colony forming units (CFUs) were observed from samples (including the initial sample) after four days of incubation on Colombia blood agar plates. This suggests that *H. pylori* is killed soon after exposure to gentamycin *in vitro* and within the two-hr period of the gentamycin protection assay.

The effect of removing FBS from the medium for 4 hrs was examined separately in both AGS cells and *H. pylori*. No morphological differences in the cells after 4 hrs incubation was seen using light microscopy, irrespective of the presence of 10 % FBS in the F12 medium. Bacteria were quantified via serial dilution for both conditions. Over two experiments, the average recovery from medium with FBS was 1.9×10^7, whereas the average recovery from medium without FBS was 9.2×10^6, indicating a 62 % drop in the number of culturable bacteria when FBS was removed from the medium over a 4 hr period.

The ability of various buffers to lyse AGS cells and their potential toxicity to *H. pylori* was tested. Lysis buffers, which included 0.5 % saponin in PBS, 0.05 % saponin in PBS, 0.25 % NaDoc, 1 % Nonidet P40 and H_20 adjusted to pH 11 with NaOH, were added to AGS cells and the cells visually examined for lysis at 5 min intervals. Treatment with Na-DOC and Nonidet P40 resulted in almost complete lysis within 5 min whereas treatment with 0.5 % saponin and pH-adjusted H_20 resulted in almost complete lysis within 10 min. After 10 min, unlysed cells were still evident in the 0.05 % saponin treatment.

To test potential toxicity of lysis buffers, *H. pylori* were suspended in medium (F12 medium + 10 % FBS) or lysis buffer. Samples were taken over regular intervals and cultured on blood agar plates. Bacteria remained viable for at least 20 mins in all lysis buffers except for 0.25 % NaDoc and 1 % Nonidet P40, which killed the bacteria within 5 min (Table 3.1).

Table 3.1: *H. pylori* viability after incubation with lysis buffers.

Incubation Period	Medium control	0.05 % Saponin	0.5 % Saponin	0.25 % NaDoc	1 % Nonidet P40	pH-adjusted H_20
5 min	lawn	lawn	lawn	0[1]	0[1]	lawn
10 min	lawn	lawn	lawn[1]	0[1]	0[1]	lawn
20 min	lawn	lawn	lawn[1]	0[1]	0[1]	lawn

(1) Small clear spots on the blood agar plates suggest some blood cell lysis.

To quantify bacterial survival, this assay was repeated once using 0.5 % saponin and H_20 pH 11 lysis buffers and serial dilutions of the bacteria were plated after 20 min of treatment (Table 3.2). The 0.05 % saponin buffer was not retested in this assay because of the observation that it failed to effectively lyse AGS cells. Treatment of *H. pylori* with 0.5 % saponin resulted in similar recoveries as the medium-only control samples. In contrast, there was a notable drop in viability after treatment with pH-adjusted H_20. From this, the 0.5 % saponin was chosen for cell lysis during the gentamycin protection assay.

Table 3.2: Quantification of *H. pylori* viability after incubation with lysis buffers.

Lysis Buffer	Bacterial Recovery[1]
Medium control	1.1×10^7
pH-adjusted H_20	1.0×10^6
0.5 % Saponin	1.6×10^7

(1) Results are from a single experiment calculated from CFUs at high dilution factors.

3.3.1 Effect of Lactoferrin on Internalization in FBS-mediated Iron-limiting Conditions

AGS cells are routinely cultured in F12 nutrient medium supplemented with 10 % FBS, which contains iron. To create iron-limiting conditions, cells were cultured in F12 medium without the addition of FBS. Partially saturated Lf from human milk was added to both iron-replete and iron-limited media. Briefly, AGS cells were incubated with *H. pylori* for 4 hrs, washed, and incubated an additional 2 hrs with gentamycin to kill any external bacteria. The cells were then lysed and serial dilutions of the lysate were plated on blood agar plates to determine the number of viable bacteria, presumed to be intracellular.

There was a large degree of day-to-day variation in the frequency of internalization. To mitigate this, the frequency of internalization was normalized to the positive (iron-replete) control of each experiment (Table 3.3). The internalization frequency was also averaged across all experiments for each condition to give an indication of the number of bacteria entering AGS cells (Table 3.3). Because the frequency of internalization was not normalized, these results do not necessarily correspond to percent of control, instead being skewed in favor of experiments with larger overall frequencies of internalization. All comparisons across conditions were done with the normalized data.

The frequency of internalization was low in all conditions, with 0.031 % to 0.66 % of the bacteria added to the AGS cells recovered during the gentamycin protection assay (Table 3.3). The number of intracellular bacteria was found to be significantly higher when the assay was carried out in iron-replete medium (p-value <0.001); the removal of FBS from the medium reduced internalization almost 10-fold (Figure 3.1). This decrease in internalization may be due to iron-limitation from removal of FBS, loss of other nutrients found in FBS, and/or changes in bacterial viability, given

that recovery of culturable bacteria from medium without FBS after 4 hrs was reduced by 62 %.

The addition of hLf (1 mg/ml, partially iron-saturated) was associated with an apparent reduction in *H. pylori* internalization of AGS cells under iron-replete conditions, and increased internalization in iron-limiting conditions. However, these differences only reached statistical significance at the 0.22 and 0.18 level.

Table 3.3: Effect of human lactoferrin on the internalization of *H. pylori* into gastric epithelial cells, without FBS as an iron-source

Lf	Medium[1]	% Internalization[2]	% of Positive Control[3]
Control (none)	Iron-replete	0.66 ± 0.61	100
none	Iron-limiting	0.081 ± 0.076	10 ± 1.0
hLf[4]	Iron-replete	0.054 ± 0.020	56 ± 29
hLf	Iron-limiting	0.031 ± 0.018	38 ± 33

(1) Iron-replete and iron-limited media defined as F12 nutrient medium with and without 10 % FBS, respectively.
(2) Mean internalization frequency, un-normalized. Calculated as the percent of inoculum recovered from AGS cells during a gentamycin protection assay. Mean of three experiements ± SE.
(3) Percent of internalization normalized to the iron-replete positive control for each experiment. Mean of three experiments ± SE.
(4) Internalization measured in the presence of 1 mg/ml of partially iron-saturated human lactoferrin..

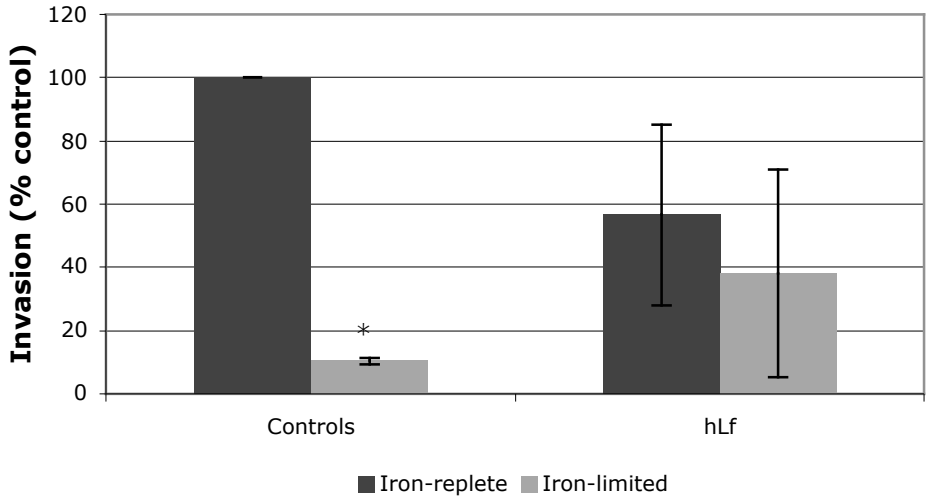

Figure 3.1: Reduction of *H. pylori* internalization into gastric epithelial cells in medium without FBS, and possible mediating effects of human lactoferrin. The ability of *H. pylori* 690190 to invade AGS gastric epithelial cells was tested in iron-replete (with addition of 10 % FBS) and iron-limiting (without FBS) F12 medium, with and without the addition of 1 mg/ml partially saturated hLf. Internalization is expressed as percent of positive control (iron-replete medium). Results are the mean ±SE of three individual experiments. *, results are statistically significantly different from iron-replete control ($p < 0.05$ by Student T-test).

3.3.2 Effect of Lactoferrin on Internalization in DE-mediated Iron-limiting Conditions

An alternative system of iron limitation was used in an attempt to clarify the effect of Lf on internalization. Here, 10 % FBS was routinely added to the F12 medium and iron-limitation was created by the addition of 20μM DE, thus avoiding any possible confounding factors involved in removing FBS from the medium. Partially iron-saturated bLf or hLf (1 mg/ml) was added to each condition.

As before, the frequency of internalization of *H. pylori* was found to be low across the conditions tested, ranging from 0.045 % to 0.28 % of the initial inoculum (Table 3.4). Again, the unnormalized internalization

frequency was provided to show that the overall number of bacteria getting into the AGS cells. For comparison across conditions, the number of internalized bacteria in the different conditions was normalized to the iron-replete control in each experiment (Table 3.4).

Table 3.4: Effect of bovine and human lactoferrin on the internalization of *H. pylori* into epithelial cells under iron-limiting conditions.

Lf	Medium[1]	% Internalization [2]	% of Positive Control[3]
Control (none)	Iron-replete	0.055 ± 0.017	100
none	Iron-limited	0.010 ± 0.0037	27 ± 13
bLf[4]	Iron-replete	0.180 ± 0.098	280 ± 170
bLf	Iron-limited	0.045 ± 0.018	91 ± 30
hLf[4]	Iron-replete	0.17 ± 0.056	240 ± 100
hLf	Iron-limited	0.28 ± 0.14	480 ± 250

(1) Iron-limited and iron-replete media defined as F12 nutrient medium with 10 % FBS, with and without 20 μM DE respectively
(2) Mean internalization frequency, un-normalized. Calculated as the percent of inoculum recovered from AGS cells during a gentamycin protection assay. Mean of four experiments ± SE.
(3) Percent of internalization normalized to the iron-replete positive control for each experiment. Mean of four experiments ± SE
(4) Internalization measured in the presence of 1 mg/ml of partially iron-saturated human lactoferrin

The use of DE, which chelates free iron in the medium (Bland *et al.* 2004), was associated with a significant decrease in the frequency of internalization of *H. pylori* into epithelial cells (p-value<0.001) (Figure 3.2). Increased internalization of *H. pylori* was evident with the addition of bLf and hLf to both iron-replete and iron-limiting conditions; this effect is most notable with supplementation of iron-limiting conditions with hLf (Figure 3.2). The effect of adding Lf to iron-limited medium was only significant to the p=0.064 (bLf) and p=0.085 (hLf) levels, which means the null hypothesis that Lf has no affect on internalization in iron-limited conditions cannot be rejected.

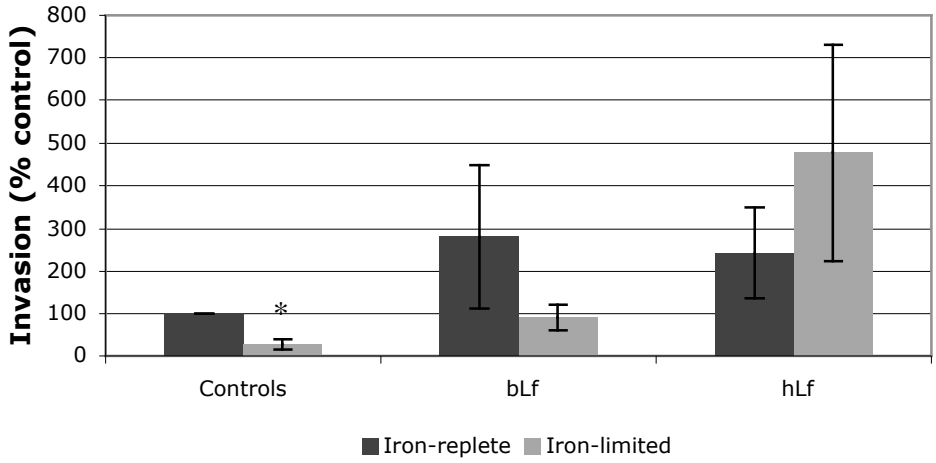

Figure 3.2: Possible increase of *H. pylori* internalization into gastric epithelial cells with the addition of lactoferrin. The ability of *H. pylori* 690190 to invade AGS gastric epithelial cells was tested in iron-replete and iron-limiting (with 20 μM DE) F12 medium, with and without the addition of 1 mg/ml partially saturated bLf or hLf. Internalization is expressed as percent of positive control (iron-replete medium). Results are the mean ±SE of three individual experiments. *, results are statistically significant from iron-replete control ($p < 0.05$ by Student T-test).

3.3.3 Effect of Denatured Human Lactoferrin on Internalization in DE-mediated Iron-limiting Conditions

The increase in internalization seen with the addition of hLf may be attributable to the presence of Lf-bound iron in the assay. To test this hypothesis, the internalization assay was repeated under iron-limiting condition (F12 medium + 10 % FBS + 20 μM DE) with or without the addition of 1 mg/ml of denatured hLf.

As previously, internalization of *H. pylori* strain 60190 into AGS cells was significantly reduced under iron-limiting conditions (p-value<0.05) (Table 3.5). However, the addition of dhLf had the opposite effect of hLf and instead significantly reduced *H. pylori* internalization of AGS cells under iron-replete conditions (p-value<0.05). In contrast, no

significant difference in the frequency of bacterial internalization was observed under iron-limiting conditions, with or without the addition of dhLf (Figure 3.3). Nor was the decrease seen with the addition of dhLf significantly different than the increase seen with hLf.

Table 3.5: Effect of denatured human lactoferrin on internalization of *H. pylori* into gastric epithelial cells under iron-limiting conditions.

Lf	Medium[1]	% Internalization[2]	% of Positive Control[3]
Control (none)	Iron-replete	2.6 ± 1.1	100
none	Iron-limited	0.99 ± 0.28	48 ± 17
Denatured hLf[4]	Iron-replete	1.4 ± 0.44	61 ± 13
Denatured hLf	Iron-limited	0.66 ± 0.16	31 ± 7

(1) Iron-limited and iron-replete media defined as F12 nutrient medium with 10 % FBS, with and without 20 μM DE respectively

(2) Mean internalization frequency, un-normalized. Calculated as the percent of inoculum recovered from AGS cells during a gentamycin protection assay. Mean of three experiments ± SE.

(3) Percent of internalization normalized to the iron-replete positive control for each experiment. Mean of three experiments ± SE

(4) Internalization measured in the presence of 1 mg/ml of partially iron-saturated human lactoferrin

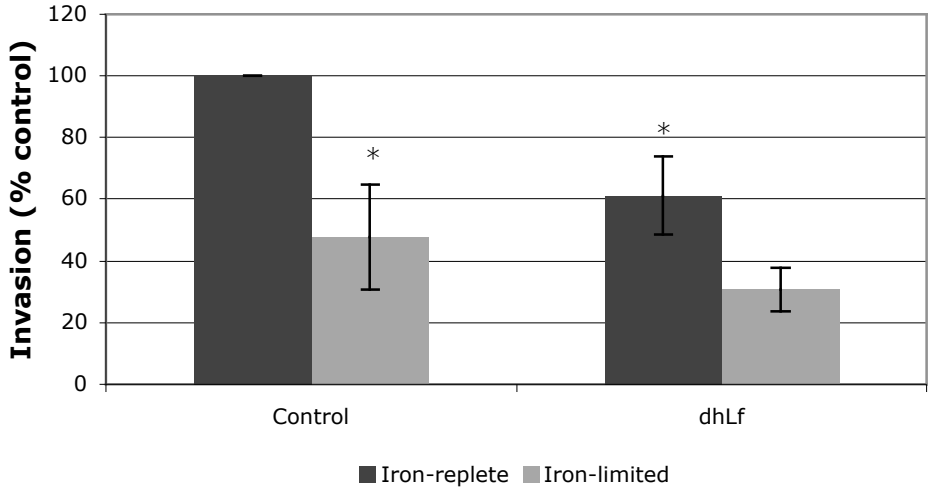

Figure 3.3: Decreased internalization of *H. pylori* into gastric epithelial cells with the addition of denatured human lactoferrin. The ability of *H. pylori* 690190 to invade AGS gastric epithelial cells was tested in iron-replete and iron-limiting (with 20 μM DE) F12 medium, with and without the addition of 1 mg/ml denatured hLf. Internalization is expressed as percent of positive control (iron-replete medium). Results are the mean ±SE of three individual experiments. *, results are statistically significant from iron-replete control ($p < 0.05$ by Student T-test).

It is interesting that internalization was significantly reduced in iron-replete conditions with the addition of dhLf. During the assay, aggregations of dhLf were apparent on the AGS cells even after washing. These large conglomerations of dhLf on the cell surface may have blocked surface sites necessary for bacterial internalization. Alternatively, the aggregates may be cytotoxic, as many aggregrates may be (Bucciantini *et al.*, 2002).

3.3.4 Effect of Lactoferrin on H. pylori Adhesion to AGS Cells under Iron-Limiting Culture Conditions

Internalization is often affected by the ability of the bacteria to adhere to host cells. To see if a change in internalization correlated with a

change in adhesion, an adhesion assay was performed with bLf and hLf in DE-mediated iron-limiting conditions. The adhesion assay was essentially identical to the gentamcyin protection assay, where AGS cells were infected with *H. pylori* for 4 hrs. Instead of incubating with gentamycin to kill extracellular bacteria, the AGS cells were gently washed before lysis to recover cell-associated (internal or external) bacteria.

The frequency of adhesion to epithelial cells was found to be higher than the frequency of internalization, ranging from 1 % to 13 % across conditions (Table 3.6).

Table 3.6: The frequency at which *H. pylori* adheres to gastric epithelial cells with the addition of lactoferrin.

Lf	Medium[1]	% Adhesion [2]	% of Positive Control[3]
Control (none)	Iron-replete	2.7 ± 2.1	100
none	Iron-limited	1.0 ± 0.89	30 ± 5.7
bLf[4]	Iron-replete	6.4 ± 3.5	510 ± 370
bLf	Iron-limited	13 ± 13	240 ± 160
hLf[4]	Iron-replete	9.1 ± 7.6	300 ± 46
hLf	Iron-limited	6.3 ± 5.7	150 ± 51

(1) Iron-limited and iron-replete media defined as F12 nutrient medium with 10 % FBS, with and without 20 μM DE respectively

(2) Mean internalization frequency, un-normalized. Calculated as the percent of inoculum recovered from AGS cells during a gentamycin protection assay. Mean of three experiments ± SE.

(3) Percent of internalization normalized to the iron-replete positive control for each experiment. Mean of three experiments ± SE

(4) Internalization measured in the presence of 1 mg/ml of partially iron-saturated human lactoferrin.

The adhesion of *H. pylori* to AGS cells was observed to be significantly higher in iron-replete medium (p-value<0.001), correlating with the internalization assay results under these culture conditions. The addition of both bLf and hLf led to an appreciable increase in adhesion,

irrespective of culture conditions, but again failed to reach significance at p=0.05 (Figure 3.4).

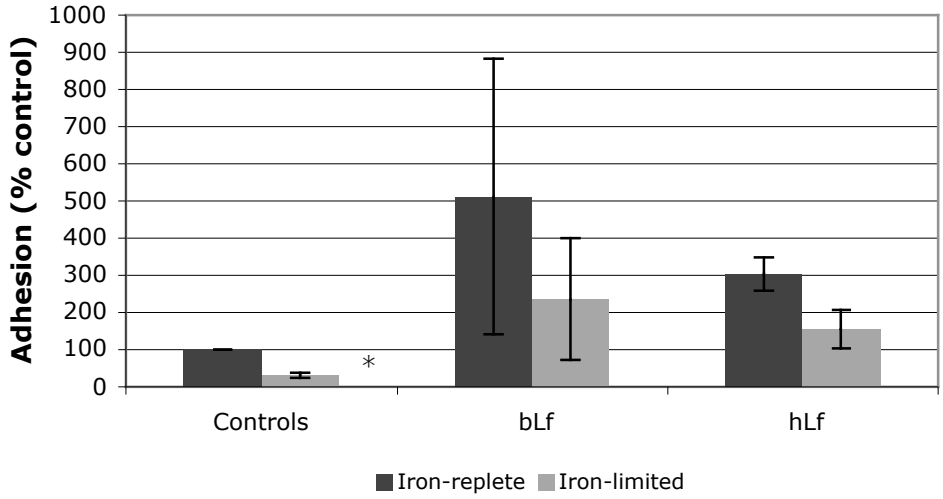

Figure 3.4: Adhesion of *H. pylori* to gastric epithelial cells: possible increase with the addition of lactoferrin. The ability of *H. pylori* 690190 to adhere to AGS gastric epithelial cells was tested in iron-replete and iron-limiting (with 20 μM DE) F12 medium, with and without the addition of 1 mg/ml partially saturated bLf and hLf. Internalization is expressed as percent of positive control (iron-replete medium). Results are the mean ±SE of three individual experiments. *, results are statistically significant from iron-replete control ($p < 0.05$ by Student T-test).

3.3.5 Analysis of the Statistical Power of the Internalization and Adhesion Assays

A power analysis was used to determine how many additional replicas would be needed to achieve significance in the internalization and adhesion assays performed here. Measured effect size (d) was calculated between the different conditions, and used during a post-hoc power analysis to determine the statistical power of each condition (Table 3.7). Statistical power is the probability of rejecting a null hypothesis when it is false and should be rejected. A low power implies that there is a higher likelihood that there actually is a difference between conditions, even when the null hypothesis cannot be rejected. Because a number of our

conditions did not reach significance, this tells us whether or not, given our data, we may be able to reject our null hypothesis with additional trials. The number of additional replicas needed to detect other effect sizes at the $p<0.05$ level, given the degree of variation seen in the original data set, was determined with an a-priori power analysis.

The effect size was calculated from the mean and standard deviation of two populations, such that either a large difference in the means of the two populations and/or a small standard deviation in the data increase the effect size. If an experiment done four times, as was the internalization assay, provides a large effect size but is not statistically significant, it may mean the actual effect size between the two conditions is smaller and needs more replicas before the data is at the significant level. The number of additional replicas needed to detect effect sizes of 0.2, 0.5, and 0.8 was calculated (Table 3.7). As previously defined by Cohen (1977), a small effect size is 0.2, a medium effect size is 0.5, and a large effect size is 0.8.

Table 3.7: Additional replicas required for detecting specific effect sizes at the p<0.05 level

			small	medium	large
	d[1]	Power[2]	0.2[3]	0.5[3]	0.8[3]
Internalization Assay					
IR, IR+bLf[4]	0.88	0.30	53	6	1
IR, IR+hLf	1.1	0.48	81	11	3
IL, IL+bLf	0.73	0.23	36	4	0
IL, IL+hLf	1.5	0.57	150	21	7
Adhesion Assay					
IR, IR+bLf	0.91	0.24	36	5	1
IR, IR+hLf	3.6	0.97	520	81	31
IL, IL+bLf	4.8	0.99	55	8	2
IL, IL+hLf	2.0	0.63	170	25	9

(1) Effect size (d) was calculated from standard deviations and means of conditions.
(2) Power was calculated from effect size and number of replicas performed with a post-hoc power analysis. Statistical power is defined as the probability of rejecting the null hypothesis when it is false and should be rejected.
(3) Number of additional replicas required calculated from statistical power using an a-priori power analysis, where 0.2 is a small effect, 0.5 is a medium effect, and 0.8 is a large effect.
(4) Comparisons made between internalization and adhesion in iron-replete (IR) and iron-limited (IL) conditions with or without the addition of 1 mg/ml of partially saturated bLf or hLf

Thus, to detect a large effect size of 0.8 in the internalization assay, additional replicas (up to 7) would be needed, depending on the conditions. To detect a large effect size in the adhesion assay, additional replicas (1-31) would be needed. If the actual effect size between any two conditions is greater than 0.8, even fewer replicas may suffice. For example, the calculated effect size between internalization in iron-limiting conditions with and without the addition of bLf is lower than 0.8, suggesting that the actual effect size is medium (Table 3.7, row 3). If it is as low as 0.5, four additional replicas would be needed. If it is between 0.73 and 0.5, then fewer replicas should give significant data.

The very high number of replicas (31) needed for an effect size of 0.8 between adhesion in iron-replete conditions with and without the addition of hLf may suggest two things. For one, the actual effect size is quite a bit larger than 0.8. Alternatively, the power for these conditions was very high, suggesting there is not a large chance that there actually is a difference between the conditions. The degree of variability between adhesion in those conditions may be too great to expect significant results without a very large, possibly impractical, number of replicas. Similarly, if any of the actual effect sizes between the conditions were small (0.2), the number of replicas needed to get significant results would be prohibitive, up to 520.

3.4 Discussion

Lactoferrin has been shown to have an inhibitory effect on the internalization of a number of facultative intracellular bacteria. Yet to date, little work has been done on species that are adapted to high levels of Lf and potentially express Lf binding proteins. Bacteria that can bind Lf, such as *H. pylori*, may be able to adhere to and invade eukaryotic cells at higher frequencies by directly binding Lf bound to epithelial cell surfaces.

Here, we tested the ability of *H. pylori* strain 60190 to invade AGS cells, and looked at how iron-limitation, bLf and hLf affected this internalization. The initial aim of this study was to see if Lf had a different effect on *H. pylori* internalization than has been seen in other bacteria, possibly via interactions with an LBP. LBPs are typically induced by iron-limiting conditions, so internalization assays were performed in both iron-replete and iron-limiting conditions.

3.4.1 Frequency of Internalization and Adherence of H. pylori into AGS cells

In this study, the overall frequency of internalization of strain 60190 into AGS cells was low but within levels reported elsewhere for other *H. pylori* strains of the same type (Type 1). From 0.0006-0.007 of the initial 60190 inoculum was recovered from AGS cells grown in medium containing 10 % FBS (iron-replete conditions). In comparison the literature values for internalization frequency for other Type 1 strains, in conditions similar to those used here, range from 0.00006 to 0.03, with one at 0.15 (Peterson *et al.* 2000, 2001, Kwok *et al.* 2002) (Table 1.3).

The frequency of adhesion of *H. pylori* strain 60190 to epithelial cells in iron-replete conditions was 0.03, much higher than the internalization frequency under the same conditions. The adhesion assay used here was based on similar assays designed to assess the role of Lf in

bacterial adhesion and internalization (Di Biase *et al.* 2004, Longhi *et al.* 2004, Superti *et al.* 2005). However, because this assay involves quantifying bacteria after cell lysis, both adherent and intracellular bacteria were detected. Thus it is more a measure of total cell-associated bacteria. In preliminary experiments, where adhesion and internalization assays were performed on the same day, numbers of adherent bacteria were found to be greater than invasive bacteria by a factor of 10 or more (data not shown).

With some facultative intracellular bacteria (e.g. *Yersinia* spp.), the majority of bacteria that adhere become internalized, resulting in a lower frequency of adherence than internalization (Superti *et al.* 2005) (see Tables 1.1, 1.2). In contrast, these results suggest that a larger number of *H. pylori* are adhering to AGS cells and that only a small proportion of these bacteria invade. This is consistent with *H. pylori* reportedly having a much lower propensity to invade epithelial cells (Peterson and Krogfelt 2003, Dubois and Boren 2007).

3.4.2 Effect of FBS on the Frequency of Internalization of H. pylori into AGS Cells

Iron-limiting conditions were initially created by not adding FBS (which contains free iron) to F12 nutrient medium. Removal of FBS from the medium resulted in a 10-fold drop in internalization. Along with iron, fetal bovine serum contains many factors necessary for growth of *H. pylori* and cultured cells. While AGS cells incubated for 4 hrs in medium without FBS did not reveal morphological signs of stress, a decline in *H. pylori* growth over this time period was observed, and may account for some of the decrease in internalization seen here.

The effect of FBS on internalization, however, may be independent of iron-limitation and/or AGS cell and bacteria viability. While FBS is iron-rich, this iron can be complexed to proteins such as transferrin, and

thus be unavailable for bacterial use (Worst *et al.* 1995). Furthermore, FBS contains protein factors that are believed to aid adherence and internalization by *H. pylori* (Peterson *et al.* 2000). Similar to results presented here, Peterson *et al.* reported a higher frequency of internalization when 10 % FBS was present (Peterson *et al.* 2000). Interestingly, the effect was eliminated when the FBS was treated with proteinase K, suggesting that the active component was a protein (Peterson *et al.* 2000).

FBS is also reported to modulated adhesion and internalization in other species of invasive bacteria. Two serum factors found within FBS, vitronectin and fibrinogen have already been shown to mediate engulfment of *N. gonorrhoaea* and *S. pyogenes*, respectively (Cue and Cleary 1997, Duensing and van Putten 1997). Vitronectin in particular facilitates $\alpha_v\beta_5$-integrin-mediated uptake by direct binding with *N. gonorrhoaea* (Kwok *et al.* 2002). *H. pylori* also binds vitronectin, and is believed to exhibit integrin-mediated internalization (Kwok *et al.* 2002).

Human Lf was added to F12 medium with FBS and without FBS. There was an apparent increase in internalization with the addition of hLf to medium without FBS, and an apparent decrease with the addition to medium with FBS, though neither was statistically significant. However, the addition of hLf did seem to confound the large decrease in internalization seen with the removal of FBS, with internalization in both conditions being similar when hLf was present. Thus, hLf may be able to mediate the loss of protein factors that aid in *H. pylori* adhesion and internalization, binding both the cell surfaces and *H. pylori* in a manner similar to that reported for components of FBS.

3.4.3 Reduction of Adherence and Internalization in Iron-Limiting Conditions

In an attempt to clarify the effect of Lf on internalization, without the possible confounding factors involved in removing FBS from the medium, another system of iron-limitation was used. Here, FBS was added to F12 medium and iron-limitation was created by the addition of 20 μM DE.

Reducing the level of available iron in the medium significantly reduced the frequency of bacterial adherence and internalization, to about 30 % of the iron-replete control. Because adherence is a necessary step in internalization, a similar reduction in the number of invading bacteria was not unexpected.

Bacteria grown for 4 hrs in iron-limited F12 medium showed no decrease in growth (measured by optical density), but did display a different phenotype, with up-regulated expression of a 70 kDa band after 4 hrs of culture in iron-limiting medium (Chapter 2). This raises the possibility that iron-associated phenotypic changes may be modulating the ability of *H. pylori* to adhere to the host cell. Iron-limiting conditions, for instance, have been shown to decrease expression of Lewis antigens (Keenan *et al.* 2008), components of LPS that mediate adhesion and internalization of *H. pylori* (Lozniewski *et al.* 2003).

3.4.4 Increase in Adhesion and Internalization in the Presence of Bovine Lactoferrin

The addition of bLf resulted in an apparent increase in adhesion and internalization, although these differences failed to reach statistical significance. Additional replications of this assay are likely to confirm that these interesting trends are indeed significant.

As before, changes in the viability of bacteria can affect internalization and adhesion rates. Most work suggests that *H. pylori* is not

able to use iron from bLf for growth (Husson *et al.* 1993, Dial *et al.* 1998), and may in fact be inhibited by it, suggesting that in increase in adhesion and internalization is not likely to be due to an increase in growth.

It is interesting that adhesion and internalization were increased to a similar degree across conditions, irrespective of iron-limitation. It has been suggested that the putative *H. pylori* LBP has low affinity for bLf (Dhaenens *et al.* 1997). If the binding of bLf to an LBP were increasing adhesion to AGS cells, we would expect to see even more bacterial adherence in iron-limiting conditions when production of LBPs is upregulated.

Like hLf, bLf can bind elements of bacteria and mammalian cell surfaces (Valenti *et al.* 2005), and this non-specific binding could lead to an increase in adhesion and thus internalization that is independent of iron levels or an iron-regulated *H. pylori* LBP.

3.4.5 Iron-related Increases in Adhesion and Internalization in the Presence of Human Lactoferrin

The addition of hLf led to an apparent increase in both internalization and adhesion, though these effects were not statistically significant. As with bLf, it is difficult to make robust statements about the effect of hLf on adhesion and internalization without additional quantitative data, but some trends can be identified.

The original hypothesis was that hLf might increase the ability of *H. pylori* to adhere to and invade human epithelial cells, by binding both epithelial cell surfaces and an *H. pylori* LBP. The addition of hLf increased adhesion to a similar degree in both iron-replete and iron-limiting conditions, analogous to what was seen with the addition of bLf. Internalization with hLf was likewise increased in iron-replete conditions. A substantially larger increase in internalization, but not adhesion, was

seen in iron-limiting conditions. If internalization in iron-limiting conditions were mediated solely by an increase in cell binding, we would expect a more correlative increase in adherence and internalization. This raises the question of whether the altered bacterial phenotype observed under iron-limiting conditions includes upregulation of a putative LBP.

It is possible then, that Lf slightly increases the number of bacteria that adhere in iron-limiting conditions, but greatly increasing the likelihood of those that adhere to be internalized. This would normally be associated with a decrease in the number of bacteria on the cell surface, as more are being internalized, but would not be reflected with the adhesion assay used here, which measured total cell-associated bacteria (Figure 3.5).

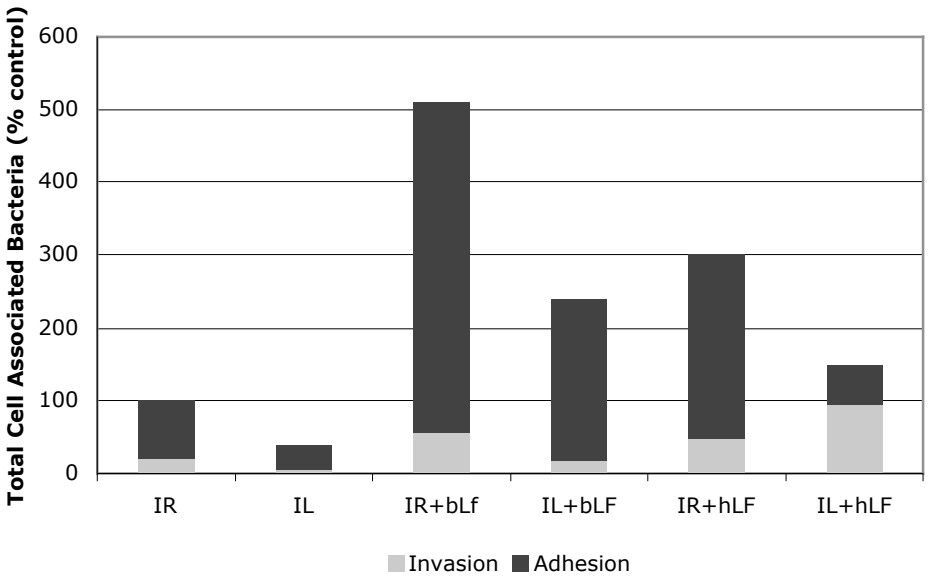

Figure 3.5: Schematic of total cell-associated *H. pylori*, including invasive and adherent bacteria. The frequency of internalization and adhesion has been generalized, to the total cell associated bacteria in iron-replete (IR) conditions reported as 100 %.

The increased internalization seen in iron-limiting conditions could be due to an increase in the production of cellular receptors for binding and

internalizing Lf. Acquiring iron is not considered a major purpose for Lf-uptake outside of the neonate intestine (Scarino *et al.* 2007), though there is evidence for iron-regulated Lf uptake in hepatocyte and myeloid cell lines (Mikogami *et al.* 1995, McAbee and Ling 1997, Olakanmi *et al.* 2002). A reduction in intracellular iron stores increases both binding and iron-uptake from Lf in hepatocytes, probably due to upregulation of the Lf receptor (Mikogami *et al.* 1995).

H. pylori may be binding the cell-bound Lf via an LBP, or through non -specific binding via the bacterial outer membrane. One way to test the relative importance of Lf binding to cellular or bacterial surfaces would be incubating both AGS cells and *H. pylori* with Lf before the infection step of the internalization assay, as reported elsewhere (Duensing and Putten 1997, Valenti and Antonini 2005). It would further need to be ascertained whether or not AGS cells exhibit iron-regulated uptake of hLf.

The internalization assay was repeated with denatured hLf (dhLf), based on the hypothesis that denaturation is likely to interfere with receptor-mediated binding, as well as releasing Lf-bound iron to the medium, allowing *H. pylori* access to it independent of any LBP.

Internalization in the presence of dhLf in iron-limiting conditions was not significantly different than internalization without dhLf in iron-limiting conditions, with a slight (and non-significant) decrease apparent. This suggests that an increase in iron in the medium is not sufficient to account for the increase in internalization seen with hLf. These data also suggest that a natively folded hLf is necessary for the increase in internalization seen here.

3.4.6 How these Data Compare to the Effect of Lactoferrin on Internalization in other Species

The majority of studies have found that both bLf and hLf reduced the ability of bacteria to invade host cells, either through binding or

degrading proteins necessary for internalization (Valenti and Antonini. 2005, Ward *et al.* 2005). There is evidence of increased adhesion of *Yersinia* spp. to epithelial cells in the presence of bLf, although no increase was seen in internalization and no mechanism for the increase was postulated (Di Biase *et al.* 2004). In this study, the presence of either bLf or hLf did not decrease either internalization or adhesion. Instead, both bLf and particularly hLf appeared to increase adhesion and internalization, though not to the statistical level.

3.4.7 Implications for Lactoferrin-mediated Effects In vivo

The majority of information we have relating to *H. pylori* internalization is from cell-based assays, which typically report a greater frequency of internalization than is apparent *in vivo* (Peterson and Krogfelt 2003). To better understand how *H. pylori* invades *in vivo*, and whether this internalization is biologically relevant, future work that more precisely replicates *in vivo* conditions is needed. The increase in internalization seen with hLf could be incidental, or could be relevant *in vivo* for bacterial persistence and pathogenicity. Internalization of *H. pylori* may, for example, affect the ability of the bacteria to persist in the host despite treatment with antibiotics.

A correlation has already been suggested between an FBS-mediated increase in internalization *in vitro*, and internalization *in vivo*. *H. pylori* would be expected to be bathed in serum factors in areas such as ulcerated epithelium, possibly explaining why larger numbers of intracellular *H. pylori* are found *in vivo* at the edges of active duodenal ulcers (Peterson *et al.* 2000). The stomach is a high hLf environment and both increasing quantities of Lf and increasing internalization of *H. pylori* has been reported in cases of severe gastritis (Chan 1992, Wen *et al.* 2004).

Many of the effects seen here did not reach statistical significance. A power analysis was performed to determine how many additional

replicas would be needed to achieve results statistically significant to the $p<0.05$ level. Assuming that Lf has a medium to large effect, the power analysis suggested that significant results could be detected after one to four additional replicas of the internalization assay with bLf, and seven additional replicas with hLf. However, the actual effect size of adding Lf may be higher than 0.8, and thus even fewer replicas may be needed to get statistical confirmation. These additional experiments would allow us to establish if Lf is capable of significantly increasing internalization and adhesion of *H. pylori*.

This work, then, provides preliminary data that will be used as a platform for future research. Should the increase in internalization by bLf and especially hLf seen here hold through in future work, this would be markedly different for what has been seen in other species. Future research could then be done on the mechanism by which hLf is acting on internalization, from increased binding via *H. pylori* LBPs to increased Lf cycling in AGS cells. Eventually, this might lead to more insight into how internalization occurs *in vivo* in high hLf conditions.

Chapter 4

The Presence of Lactoferrin Binding Sites in Bacterial Genomes: Potential Roles as DNA Vector and Transcriptional Regulator

4.1 Experimental Justification

Lf has at least one DNA binding domain able to bind specific DNA sequences (He and Furmanksi 1995). In eukaryotes, Lf is capable of upregulating expression of genes downstream of these sequences, referred to in the literature as lactoferrin response elements (LFREs) (He and Furmanksi 1995, Son *et al.* 2002, Mariller *et al.* 2007). Lf is also able to bind DNA outside of the nucleus, and has the potential to act as a vector, bringing extracellular DNA into human cells as it is translocated across the membrane (Sinogeeva *et al.* 2000).

A number of bacteria that live within mammals come into contact with Lf extensively during their lifetimes, including the stomach pathogen *H. pylori*. The possibility of Lf binding sites in bacterial genomes suggests that Lf could bind and possibly facilitate the movement of prokaryotic DNA with and between species. The initial paper identifying Lf as a transcription factor suggested it might work on targeted genes in bacteria as well (He and Furmanksi 1995).

Here we use bioinformatics tools to see if bacteria have LFREs in their genomes. Consensus sequences were made for each of the three LFREs, and 100 bacterial genomes were searched for their presence. To determine if these sequences were appearing at random, the observed occurrence of these sequences was compared to the expected occurrence of a random sequence of the same length and base composition. The presence of Lf binding sites at a greater or lesser frequency than random sequences may suggest that they have a function. The location of binding sites in

relation to downstream elements was further investigated in *H. pylori*, to determine their potential to regulate transcription.

4.2 Materials and Methods

4.2.1 Searching the Literature for Known LFREs.

The literature was searched for reported, functional LFREs via the databases Web of Science (www.isiknowledge.com) and PubMed (www.ncbi.nlm.nih.gov/pubmed/) using the terms 'Lf', 'DNA', 'DNA binding', 'transcription activation', and 'gene expression' individually or in some combination. Reported LFREs were chosen that were shown to be specifically bound by Lf, and/or promote expression of reporter genes or actual genes.

4.2.2 Generating Consensus Sequences

All LFREs known to drive gene expression, as determined above, were put into an online WebLogo generator (http://weblogo.berkeley.edu/; Schneider TD *et al.* 1990, Crooks *et al.* 2004) to generate Sequence Logos. The generated Sequence Logos were then used to derive consensus sequences, written in the FASTA format (Table 4.1).

Table 4.1: FASTA format abbreviations

A	Adenine	S	G or C (Strong interaction)
C	Cytosine	W	A or T (Weak interaction)
G	Guanine	B	G, T or C (not A)
T	Thymidine	D	G, A, or T (not C)
U	Uracil	H	A, C, or T (not G)
R	G or A (puRine)	V	G, C, or A (not, T, not U)
Y	T or C (pYrimidine)	N	A, G, C or T (aNy)
K	G or T (Ketone)	-	gap
M	A or C (aMino group)		

4.2.3 Generating Scrambled Consensus Sequences.

Each consensus sequence was scrambled 10 times with the ShuffleSeq program from EMBOSS (Rice *et al.* 2000), through the online Mobyl portal (mobyle.pasteur.fr). Sequences and scrambled sequences (scLFREs) were tested to see if they were known prokaryotic response elements using the PRODORIC prokaryotic gene expression search engine, searching the transcription factor binding site database (prodoric.tu-bs.de; Münch *et al.* 2003).

4.2.4 Searching Bacterial Genomes for LFREs and Scrambled LFREs.

LFRE and scLFRE searches were performed on 100 prokaryotic genomes using the DNA scan program from the National Microbial Pathogen Data Resource website (www.nmpdr.org**;** McNeil *et al.* 2006). Information on host, pathogenicity, genome size and GC content of each species searched was obtained from Genomes Online (www.genomesonline.org) using the GOLD search program (Liolios *et al.* 2008). The number of LFREs in each genome was normalized by genome size to determine the number of LFREs per kilo-bp to compare across species. The number of hits from the 10 scLFREs was used to calculate a

95 % confidence interval for each genome, which was then compared to the number of actual LFREs found in that genome.

4.2.5 Location of Lactoferrin Binding Sites Relative to ORFs in H. pylori

The NMPDR website was used for analyzing functional elements adjacent to reported LFRES in *H. pylori*. Because Lf binds LFREs in double stranded DNA, the nearest gene on either strand was recorded. When the LFRE fell within the gene, the distance to the nearest downstream element on either strand was also recorded. A Chi-squared test was used to analyze the frequency at which LFREs fell within genes. The distribution of LFREs relative to the nearest downstream element was analyzed in 200 bp intervals. The distribution of LFRE1b was compared to the distribution of 3 scrambles of the LFRE1b consensus sequence.

4.3 Results and Discussion

4.3.1 Searching the Literature for Known Lactoferrin Response Elements

Lactoferrin was initially reported to bind three response elements, labeled LFRE1, LFRE2 and LFRE3 (He and Furmanski 1995). Lf was able to increase transcription of a CAT reporter gene in eukaryotic cells with upstream LFREs (See Table 4.2) (He and Furmanski 1995). Two versions of LFRE1 with single base-pair mutations were also shown to drive significant levels of CAT gene expression (above 50 % of original LFRE1), and those sequences were included for further analysis.

Two genes in the human genome, IL-1β and Skp1, have functional LFREs within their promoters. Two response elements were found before the Skp1 gene, both capable of individually increasing gene expression (Mariller *et al.* 2007). They also appeared to act synergistically, with the presence of both LFREs resulting in the highest level of gene expression. Five response elements were found upstream of the IL-1β gene, and Lf was shown to stimulate expression of an IL-1β-luciferase reporter plasmid containing all five response elements in eukaryotic cell lines (Son *et al.* 2002). It was not determined which combination was necessary for transcription activation (though all were bound by Lf) and all variants were included here. One other possible response element was identified for the Rb gene, with the sequence TGCACTTGTAT in the Rb promoter (Mariller *et al.* 2007). Further work is needed to determine if the sequence is a functional lactoferrin response element, and as such was not included in further analysis.

Table 4.2: Functional lactoferrin response elements reported in the literature[1]

LFRE	Cell Type	Gene	Source
GGCACTT(G/A)C GTCACTTGCGGCAATTGC TAGA(A/G)GATCAAA ACTACAGTCTACA	K562	CAT reporter gene	He and Furmanski 1995
GGCACTTGC…(-23193)[2] GGAACTTGC…(-23129)[2] GGAACTTGC… (-21043)[2] GTCACGTGC…(-22376)[2] GGCACTGTGC…(-21348)[2]	COS-1 K562 U947	IL-1β[3]	Son *et al.* 2002
GGCACTGTAC…(-1067bp)[2] TAGAAGTCAA…(- 646 bp)[2]	HeLa MDA-MB-231 HEK 293	Skp1[4]	Mariller *et al.* 2007

(1) The databases Web of Science and PubMed were searched for literature reporting LFREs known to bind DNA and increase transcription of downstream genes.
(2) Distance between the LFRE and the start codon of the downstream gene.
(3) IL-1β is released by macrophages, mediating tissue damage and activating a cascade of cytokines, including TNF-α.
(4) S-phase kinase-associated protein, part of the Skp1/Cullin-1/F-box ubiquitin ligase complex, responsible for the ubiquitination of cellular regulators preceding proteolysis.

4.3.2 Generating Consensus sequences

The LFREs found in front of IL-1β and Skp1 were all similar to those identified by He and Furmanski. As such, all reported functional response elements were grouped according to their similarity to LFRE1, LFRE2, or LFRE3 (Table 4.3). Each group of sequences was entered into the online WebLogo program to generate sequence logos. A sequence logo represents the frequency at which certain bases occur in certain positions graphically, by relative height (Figure 4.1).

Table 4.3: Aligned input sequences for the WebLogo generator

	Input
LFRE1	GGCACTGTAC GGCACTGTGC GTCAC-GTGC GGAACT-TGC GTCACT-TAC GTCACT-TGC GGCACT-TAC GGCACT-TGC GGCACT-TGC GGAACT-TGC
LFRE2	TAGAAGATCAAA TAGAGGATCAAA TAGAAG-TCAA
LFRE3	ACTACAGTCTACA

(A) LFRE1

Sequence Logo

Consensus Sequences: **(1a) GKMACTGTRC**
(1b) GKMACTTRC

(B) LFRE2

Sequence Logo

Consensus Sequences: **(2a) TAGARGATCAA**
(2b) TAGARGTCAA

(C) LFRE3

Sequence Logo

Consensus Sequence: **ACTACAGTCTACA**

Figure 4.1: Sequence logos for lactoferrin response elements. Known functional LFREs were grouped into three similar sequences and entered into a WebLogo program to generate sequence logos. The prevalence of each base in a given position is demonstrated by relative height. Consensus sequences were derived using FASTA format, with multiple consensus sequences for elements of variable length.

Consensus sequences were built from the sequence logos for use in an online genome search program that reads FASTA format. FASTA format allows one to specify multiple combinations of bases for each position within the consensus sequence (Figure 4.1). Both LFRE1 and LFRE2 had an optional base in position 7. Because consensus sequences do not allow for alternate spacing (a specific base or no base at all), two consensus sequences were built for these LFREs, a short one for 'no-base' in position 7 and a longer one for 'this-base' in position 7 (Figure 4.1). The final base was not added to the LFRE2 consensus sequences because the final base was "any base" and should not affect the occurrence of the sequence within the genome. For convenience, the consensus sequences were labeled after the sequences they were derived from, as LFRE1a, LFRE1b, and LFRE2 etc. These labels are not meant to indicate that a given sequence occurring within a genome is necessarily functional.

Consensus sequences do not account for the relative frequency of a given base in each position, giving all possible bases in a position equal weight. Because of this, an unusual binding site can have an unduly large influence on the final consensus sequence (Wasserman and Sandelin 2004). One way that the limitations of consensus sequences can be overcome is with the use of position weight matrices (Wasserman and Sandelin 2004, Stormo 2000).

Weight matrices are used to calculate the frequency at which a specific base occurs in a specific position, a mathematical approach to the visual weighting seen in the sequence logos. This weighting appears to have biological significance, with the weight matrix 'score' of a particular sequence found to correlate with activity as a promoter (Stormo 2000).

The benefit of weight matrices diminishes with small data sets, such as the one for lactoferrin response elements. Furthermore, the focus here is more on the prevalence and location of Lf binding sites, not the

relative ability of any given found sequence to drive transcription. Were Lf shown to activate transcription in prokaryotes, and a database of functional prokaryotic Lf response elements tabulated, weight matrices could become a more reasonable choice.

The use of consensus sequences maximizes the potential number of Lf binding sites found in each genome. In this analysis, all possible lactoferrin response elements known to be bound by Lf and/or act as a regulatory element were included, even when their individual ability to drive transcription was not known, such as with the response elements found before the human IL-1β gene. This inclusivity may be biologically justified, because functional binding sites for regulatory proteins *in vivo* are often more diverse than those found *in vitro* (Shultzaberger and Schneider 1999). Alternatively, there is the danger of false binding sites that can increase the number of times a given consensus sequence occurs in a genome, including non-functional ones, and obscure potential patterns in where they fall in relation to genes.

4.3.3 Determining if and how Frequently Lactoferrin Binding Sites occur within Bacterial Genomes

The genomes of 100 bacteria were searched for the presence of all five consensus sequences with the National Microbial Pathogen Data Resource (NMPDR) genome search program, DNA scan (McNeil *et al.* 2006). Species from numerous classes were chosen. These include ones that are thought to use Lf, ones that could potentially have come into contact with Lf through pathogenesis or host colonization, and ones that are unlikely to have regular contact with Lf.

All species had at least one Lf binding site in their genome (see Appendix II for complete data tables). The vast majority of the sequences found across the genomes were LFRE1, making up 97.3 % of the total.

Every species had at least one LFRE1a or LFRE1b. The shorter LFRE1b occurred at a greater frequency than the similar but longer LFRE1a.

LFRE2 made up 2.7 % of the total LFREs found across genomes. Though a much smaller percentage of the total LFREs, all but 7 species of 100 had at least one LFRE2a or LFRE2b. Again, the shorter LFRE2b occurred at a greater frequency than LFRE2a. LFRE3 did not occur once in any of the genomes.

This is consistent with *a priori* calculations of how often a given sequence is likely to occur within a genome, depending on its length and the number of variable positions that occur. This calculation assumes an equal composition of all four bases, which is unlikely to be strictly true for any given genome, but it can provide a general idea of how often the different consensus sequences would occur relative to each other (Table 4.4).

Table 4.4: Expected occurrence of a given sequence in a genome assuming equal composition of all bases.

Consensus Sequence	Expected Occurrence (per kbp)[1]
LFRE1a	0.0076
LFRE1b	0.030
LFRE2a	0.00048
LFRE2b	0.0018
LFRE3	0.000015

(1) Calculated from the number of positions in the sequence where one base can occur x, and the number of positions where two bases can occur y, such that: $4^x * 2^y = Z$, with the sequence expected to occur once every Z bps. The inverse was taken to provide the expected occurrence of the sequence per kbp.

The total number of Lf binding sites, the sum of all three consensus sequences, varied greatly from species to species. To compare across species, the number of total Lf binding sites for each species was divided by the genome size of that organism to provide the number of binding sites per kbp. These were then averaged within each phylogenetic class represented (Figure 4.2).

The number of Lf binding sites per kbp ranged from a low of 0.041 among the Epsilon proteobacteria, to a high of 0.122 among the Actinobacteria-Actinomycetales class (Figure 4.2).

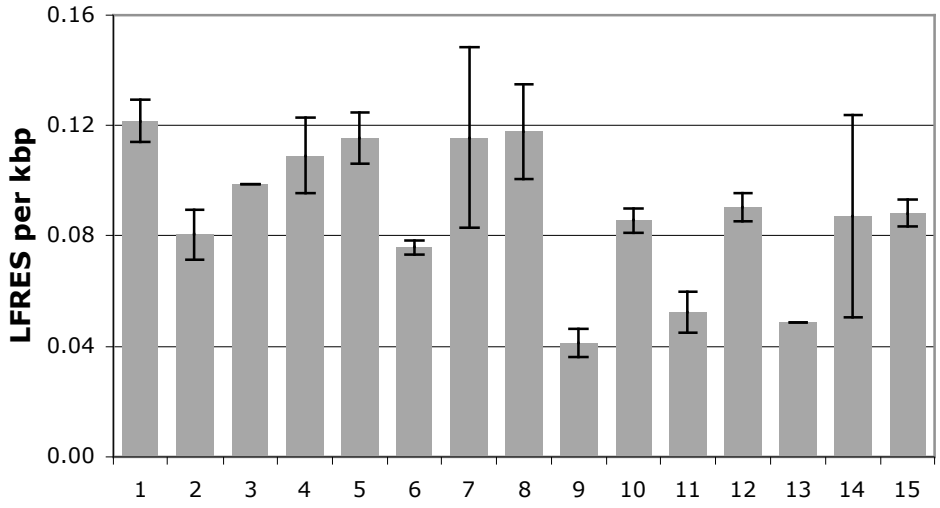

Figure 4.2: Average number of lactoferrin binding sequences per kilo base pair by kind of bacteria. A hundred genomes were searched for the presence of Lf binding sites. For each species, the total found Lf binding sites was divided by genome size to get the number of binding sites per kbp. Standard error bars represent the variation between species within that class. (1) Actinobacteria-Actinomycetales (2) Alpha Proteobacteria (3) Aquificae (4) Bacteriodetes (5) Beta proteobacteria (6) Cyanobacteria (7) Deinococcus-Thermus (8) Delta Proteobacteria (9) Epsilon Proteobacteria (10) Firmicutes-Bacilli (11) Firmicutes-Clostridia (12) Gamma Proteobacteria (13) Planctomycetes (14) Spirochaetes (15) Thermotogae

The Potential of Lactoferrin to act as a DNA Vector for Horizontal Gene Transfer

This work has demonstrated the presence of the consensus sequences and thus Lf binding sites in every prokaryotic genome searched. Lf has already been shown to bind the CpG islands common in bacterial DNA, and modulate the subsequent host immune response by inhibiting the uptake of CpG-containing DNA into cells (Britigain *et al.* 2001,

Mulligan *et al.* 2006). Yet research using Lf as a DNA vector shows that it can also facilitate the internalization of DNA with lactoferrin binding sites (Elfinger *et al.* 2007), and in some cases, transport it to the nucleus (Sinogeeva *et al.* 2000). The presence of the lactoferrin response consensus sequences in bacterial genomes may mean that Lf can bind sections of bacterial DNA, other than CpG islands, and transport DNA of bacterial origin into eukaryotic cells.

4.3.4 Analysis of Whether Sequences are Occurring at Random within Genomes

The likelihood that the Lf binding sites are occurring at random was investigated. Each consensus sequence was scrambled 10 times by the program ShuffleSeq that shuffles both nucleotides and amino acid sequences (Table 4.5). There is evidence that scrambling lactoferrin response elements eliminates their function as a transcription factor, with scrambling eradicating the ability of LFRE1 to activate gene expression (He and Furmanski 1995). All 100 species were searched for the prevalence of the scrambled sequences using the NMPDR database.

Table 4.5: Sequences for shuffled lactoferrin response elements[1]

LFRE1a	LFRE1b	LFRE2a	LFRE2b	LFRE3
GKMACKGTRC	GKMACKTRC	TAGARGATCAA	TAGARGTCAA	ACTACAGTCTACA
KACTGMKRGC	AGTKRMCCK	AGATAACRAGT	ARACTAAGTG	AATACCTTCAACG
CRCGKMAGTK	GTRAKCKMC	TCAGARAGAAT	CGTAAATRGA	GCTCCTCAAAATA
KKGRMTACGC	CAKRGKCTM	ARCGAATGAAT	ACAGATTARG	ACCCCAAGTATAT
TGRGMCCAKK	MCARKCTKG	AAATAGATCRG	ATGTCRAGAA	CACATTCAGTAAC
GRAMKKCGTC	MGTCCKRAK	TRTACGAAAAG	ATARAAGCTG	TACACGCACTATA
RKGCMGCTAK	CMCTKRKAG	AGCTAARGAAT	ATRAGGCTAA	GAATCACCTCTAA
MGKRKGCTAC	CMRGKKCTA	ATAACATGAGR	GCAAGTARTA	AACTACGATCTCA
GGMTKRKCCA	GKCATRCKM	AATTCAGRAGA	ARGATAACTG	CATGCACATCATA
CAKKRGGMTC	CMAKCGKTR	TATAAGCGRAA	CGTAAAGTRA	TCTGTACCACAAA
CTCKKGRAMG	TKCGMCAKR	AAGACGTTAAR	CAGGARTAAT	GCATTATAAACCC

(1)The five LFRE consensus sequences were shuffled on the program ShuffleSeq to generate 10 randomized sequences of same length and base composition as the original LFRE

Each of these scrambled LFREs (scLFREs), as well as the initial consensus sequences, were checked against a database of known prokaryotic transcription factor binding sites. This was to ensure that the consensus sequences were not being compared against functional sequences that may occur at a non-random frequency within the genome. None of these sequences were recorded as known transcription factor binding sites, so all were used for determining the number of scLFREs within the genomes.

However, this does not preclude unknown functions for the scrambled sequences. In fact, of the 2000-3000 transcription factors believed to be encoded in the human genome, only 123 have experimentally determined binding sites in the eukaryotic transcription binding site database, JASPAR (Zeng *et al.* 2008). Though not performed here, it would be possible to scramble the genomes of the bacteria, and tabulate how often the non-scrambled consensus sequences appear in the scrambled genome. This would still account for genome length and GC content, but would eliminate the potential problem with functional scrambled consensus sequences.

The prevalence of the 10 scrambled sequences was used to generate a 95 % confidence interval for the number of a times a given sequences of that length and base composition would be expected to appear. The confidence intervals were generated for individual genomes, thus accounting for differences in genome length and GC content across species. The observed occurrence of each consensus sequence was then compared to its confidence interval to see if they occurred outside of the expected frequency for a random sequence. When an LFRE consensus sequence appeared in a genome higher or lower than the confidence interval, it was marked with a (+) or a (-), respectively (Table 4.6; see Appendix II for complete data tables).

Table 4.6: Comparison of observed occurrences of consensus sequences within the *H. pylori* strain J99 genome to occurrence of random sequences

Consensus Sequence	Mean Occurrence of Random Sequences	95 % Confidence Interval	Observed Occurrence of Consensus Sequence	+/-
LFRE1a	21	± 8.8	1	-
LFRE1b	120	± 55	42	-
LFRE2a	2.8	± 2.9	8	+
LFRE2b	7.8	± 7.0	19	+
LFRE3	0	± 0	0	

The main observations are as follows:

- Most species (84) had at least one consensus sequence that occurred at a frequency outside of the confidence interval, though often by a small margin.
- No species was found to have all five consensus sequences at a greater or lesser prevalence than expected from the confidence interval.
- Only a few species (12) were found to have a greater or lower prevalence of both consensus sequences for an individual LFRE (e.g. both LFRE1a and LFRE1b or both LFRE2a and LFRE2b outside of the confidence interval) as was seen in *H. pylori* strain J99.

Bacterial species thought to use Lf as an iron-source were examined to see if there were any patterns of Lf binding sites occurring at a greater or lesser frequency than would be expected from the confidence intervals (Table 4.7). There were no apparent patterns across these species.

Table 4.7 Occurrence of lactoferrin binding sites in comparison to expected frequencies[1] in species thought to use lactoferrin

		LFREs				
Disease	Species	1a	1b	2a	2b	3
Respiratory infection	*Bordetella pertussis* Tohama I		+			
Ulcer, gastric inflammation	*Helicobacter pylori* 26695		-	+		
Ulcer, gastric inflammation	*Helicobacter pylori* J99	-	-	+	+	
Gonorrhea	*Neisseria gonorrhoeae* FA 1090	+				
Meningitis, septicemia	*Neisseria meningitidis* FAM18					
Mastitis	*Streptococcus uberis* 0140J			+		
Periodontal infection	*Treponema denticola* ATCC 35405			-		
Syphilis	*Treponema pallidum* subsp. pallidum str. Nichols	+	+	-		

(1) The number of LFRE consensus sequences in each genome was compared to the expected frequency (95 % confidence interval) of sequences of the same length and base composition in a given genome. LFREs that occurred at a greater or lower than expected frequency were marked with a (+) or (-), respectively.

Many of the 100 bacteria examined here could potentially come into contact with Lf, either long-term through host colonization, or more briefly as in the case of soil and water bacteria, which may enter the host via the food supply. Some of these bacteria were examined together to see if any patterns emerged (Table 4.8). Again, there appeared to be no overall pattern for an increase or decrease in LFREs from the 95 % confidence intervals. Bacteria not expected to come into regular, extended contact with Lf, such as those that regularly inhabit thermal vents, had a similar,

apparently random, occurrence of LFREs outside of the expected frequency (data not shown; refer to Appendix II for complete data tables).

Table 4.8: Occurrence of lactoferrin binding sites in comparison to expected frequencies[1] in species likely to come into contact with lactoferrin

		LFREs[1]				
Disease	Species	1a	1b	2a	2b	3
Anthrax	*Bacillus anthracis* str. A1055	+		+	-	
Food poisoning	*Bacillus cereus* ATCC 10987	+		+	-	
Food poisoning	*Bacillus licheniformis* ATCC 14580		-			
Brucellosis	*Brucella abortus* biovar 1 str. 9-941			-		
Brucellosis	*Brucella melitenus* 16M		-	-		
Pneumonia	*Burkholderia cenocepacia* AU 1054		+			
Bactaeremia	*Campylobacter jejuni* subsp. doylei 269.97	-	-			
Diarrhea, septicemia	*Chromobacterium violaceum* ATCC 12472		-	+	-	
Botulism	*Clostridium botulinum* A str. ATCC 19397	-		+		
Diphtheria, respiratory infection	*Corynebacterium diphtheriae* NCTC 13129			-	+	
Ehrlichiosis	*Ehrlichia chaffeensis* str. Arkansas		+	+		
Urinary infection, bactaeremia	*Enterococcus faecalis* V583					
None	*Escherichia coli* K12	+			-	
Diarrhea, hemorrhagic colitis	*Escherichia coli* O157:H7	+		-	-	
Meningitis	*Haemophilus influenzae*	-			+	
None	*Lactobacillus acidophilus* NCFM	-		-	-	
Opportunistic/ nocosomial infection	*Pseudomonas aeruginosa* UCBPP PA14		-			
Food poisoning; Salmonellosis	*Salmonella bongori* 12149				-	
Salmonellosis, food poisoning	*Salmonella typhimurium* LT2		-	-	-	
Pneumonia	*Streptococcus pneumoniae* 23F	-			+	

(1) LFRE consensus sequences were scrambled 10 times to generate 95 % confidence intervals for a sequence with the same length and base compositions in each genome. LFREs that occurred at a higher frequency than predicted by the confidence interval represented with a (+) and LFREs that occurred at a lower frequency than predicted represented with a (-) (see Appendix II for complete data tables).

While Lf binding sites do occasionally occur at greater or lesser frequency than would be expected for similar sequences of the same length and base composition, they appeared to do so at random. This may indicate that Lf binding sites have not been recruited or culled by natural selection. While no strong patterns emerged across these species, it is not necessary for a particular sequence to be enriched in a genome to be functional. A role for Lf in individual species, then, cannot be precluded.

Furthermore, a number of species that come into regular contact with Lf, such as *H. pylori* and *Neisseria* sp, are naturally transformable, that is they take up DNA readily from their environment (Saunders *et al.* 1999, Smeets and Kusters 2002, Hamilton and Dillard 2006). By interacting with Lf, they would also come into contact with Lf-bound DNA. *Neisseria* sp preferentially take up DNA containing a 10 bp uptake sequence frequent within their own genome, which increases the likelihood that the DNA is derived from related bacteria (Hamilton and Dillard 2006). A similar uptake sequence has not been identified in *H. pylori* (Saunders *et al.* 1999). If Lf were regularly functioning in horizontal gene transfer, areas of the genome with more Lf binding sites would more likely be transferred, with Lf binding sites acting as de facto uptake sequences.

4.3.5 Location of Lactoferrin Binding Sequences in the H. pylori Genome

The genome of *H. pylori* strain J99 was examined to see if the Lf binding sequences that occur in the genome (70 total) were in locations that suggest they could have a function in the regulation of transcription.

An initial search was conducted on the location of the consensus sequence in relation to the nearest functional element, as defined by the NMPDR database. Most (62) Lf binding sites fell within an open reading frame (ORF) (see Appendix II for complete data tables). The genome of *H. pylori* strain J99 has been completely sequenced, and the predicted

proportion of the genome believed to be outside of an ORF is 0.092 (Tomb *et al.* 1997).

A chi-squared test was used to determine if the actual frequency of a lactoferrin response element occurring outside of an ORF corresponded to the expected frequency. This resulted in a p-value of 0.50, such that the null hypothesis that there is no difference between the expected frequency and actual frequency of Lf binding sites occurring outside of ORFs cannot be rejected.

While not at a frequency greater than expected, Lf binding sites still exist mostly within ORFs. This suggests that were Lf to bind extracellular *H. pylori* DNA, it would likely be within an ORF. This would increase the chance that functional DNA is being transferred during any possible Lf mediated horizontal gene transfer.

Lf binding sites that occur within ORFs are also often relatively near to additional downstream ORFs. The distance between every Lf binding site and the nearest downstream ORF was calculated, to determine if the sequences occurred more frequently in some positions, possibly indicating functionality (see Appendix II).

Many binding sites for transcriptional activators and repressors are found near the promoter, about –35 bp from the start codon (Madigan *et al.* 2003). Cis-acting sequences that regulate transcription tend to be near to the initiation of transcription, with current data suggesting that sequences near a transcription start are more likely to be important regulators than more distant sequences (Wasserman and Sanderman 2004). Only 2 of the 70 Lf binding sites fell within 100 bps of the nearest downstream ORF.

However, regulatory elements can also occur at hundreds of base pairs away from the genes they modulate (Madigan *et al.* 2003), and indeed LFREs in the human genome have been found up to 2300 bp upstream. Most LFREs occurred within 1000 bp of the nearest downstream element; those that did not were scattered thinly up to 6000 bp away. To

see if any positions appeared to be enriched for Lf binding sites, the proportion of sequences that occurred up to 1000 bps before a gene was tabulated, in 200 bp intervals (Table 4.9).

The largest proportion of LFREs occurred between 200-400 bp upstream of the nearest element (Table 4.9). A similar pattern was seen for the two consensus sequences with the greatest number of hits within the *H. pylori* genome, LFRE1b and LFRE2b. The distribution of LFRE1b was compared to the distribution of three scrambles of the LFRE1b consensus sequences (Table 4.9, Figure 4.3).

Table 4.9: Distribution of lactoferrin binding sites at 200 bp intervals upstream of functional elements[1]

	0-200[2]	200-400[2]	400-600[2]	600-800[2]	800-1000[2]	1000+[2]
Total LFREs	0.100	0.186	0.100	0.100	0.100	0.314
LFRE1b	0.119	0.214	0.048	0.095	0.095	0.381
scLFRE1b.i[3]	0.107	0.107	0.143	0.036	0.179	0.321
scLFRE1b.ii[3]	0.163	0.143	0.122	0.102	0.163	0.429
scLFRE1b.iii[3]	0.120	0.179	0.145	0.120	0.060	0.350

(1) Distribution of LFRE consensus sequences, reported as the proportion of total LFREs that fell within that distance interval from the nearest downstream functional element.
(2) Number of base pairs between the Lf binding site and the nearest downstream functional element.
(3) LFRE1b scrambled to provide sequences of the same length and base composition.

The distribution of the scrambled sequences varied widely, yet the increase in Lf binding sites at the 200-400 bp interval in scLFRE1b.iii suggests that the distribution of LFRE1b is not unlikely for a sequence of that length and base composition.

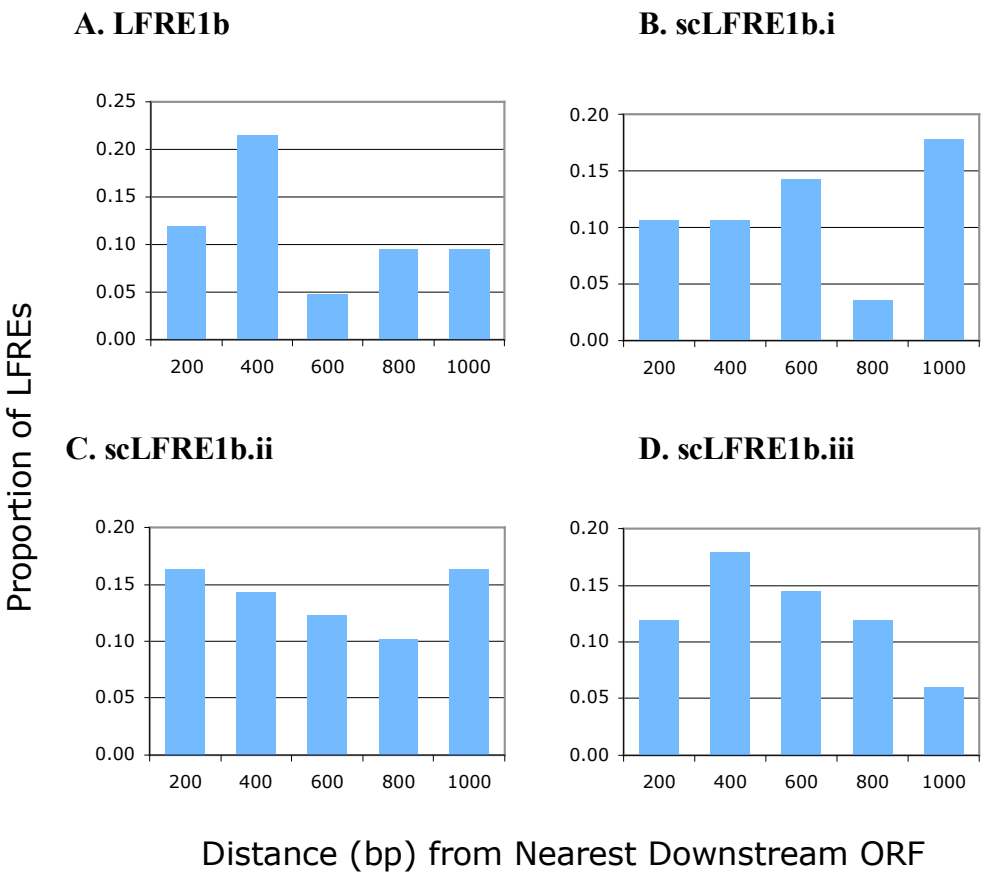

Figure 4.3: Distribution of lactoferrin binding sites at 200 bp intervals upstream of functional elements. Distribution of Lf binding sites, reported as the proportion of total binding sites within 200 bp intervals from the nearest downstream ORF.

Regulatory proteins often have affinity for sequences of DNA outside of their target sites, which can lead to false positive identifications (Stormo 2000). A large number of false positives is likely to obscure any patterns seen in the distribution of Lf binding sites. That said, the data presented here does not provide compelling evidence for Lf binding sites to act as transcriptional regulators in *H. pylori* strain J99.

4.4 Conclusion

This work showed the presence of potential Lf binding sites within a broad range of bacterial genomes. The presence of potential Lf binding sites suggests that Lf can bind bacterial DNA. If so, then it is reasonable to speculate that Lf could be a vector for uptake of this DNA just as it is for DNA of eukaryotic origin. Lf's ability to act as a DNA vector, then, may be exploited for drug and DNA vaccine delivery, but it could also increase non-specific DNA transfer. *H. pylori* undergoes a particularly high rate of natural transformation with DNA from the environment (Saunders *et al.* 1999, Smeets and Kusters 2002, Baltrus and Guillemin 2006); its ability to use lactoferrin brings it into close proximity of any DNA that might be bound to it.

Since Lf interacts with both bacterial cells and eukaryotic cells, it may shuttle DNA back and forth from prokaryotes and eukaryotes in the extracellular environment and possibly even bring the DNA into human cells. Furthermore, genes have been shown to transfer between *Salmonella enterica* within epithelial cells (Ferguson *et al.* 2002). By increasing the uptake of *H. pylori* into epithelial cells, Lf could be increasing the chances of gene transfer within the intracellular environment.

The frequency and location of Lf binding sites appear to be random, with no patterns emerging of sequences being enriched across species or of them occurring in similar positions relative to downstream ORFs in *H. pylori*. While this work provides no evidence that Lf has a role in bacterial transcription, additional laboratory based research would be needed to determine if this indeed were the case.

Chapter 5
Summary and Future Work

The aim of this study was to increase knowledge of the Lf-bacterial interface, especially at the interface of bacteria that might come into regular contact with Lf. Bacteria that are regularly exposed to large quantities of Lf would need to adapt to its antimicrobial properties, and those that adapt might even exploit it (Heinemann 2008). This is interesting as it applies both to the microbial ecology of the human body, and to issues of biosafety. Recombinant hLf is currently being produced in plants and soon may also be produced in cows and harvested from the milk (Heinemann 2008). This will likely increase the range of bacteria that are exposed, and potentially adapted, to Lf.

Our first thought was that the functions Lf is known to have in eukaryotes, such as iron absorption and transcription activation, might extend to prokaryotes and thus these are the functions most likely to be exploited. *H. pylori* was chosen as a model organism to investigate these speculations. It lives in a high-Lf environment and has previously been reported to use Lf as an iron-source. Here, the affects of Lf on *H. pylori* growth and internalization were investigated. Also, using bioinformatics tools, a number of bacterial genomes were searched for the presence of Lf binding sites, and these sites were examined in relation to Lf's potential as a DNA vector or transcriptional regulator in prokaryotes.

In this study, growth of *H. pylori* strains 60190, SS1 and Tx30a decreased in the presence of partially iron-saturated hLf. Growth of strain 60190 was also tested with fully iron-saturated hLf. In iron-limited media, growth increased to levels comparable to the iron-replete control when holo-hLf was added as an iron source. A slight increase in growth was also seen in iron-replete medium when supplemented with holo-hLf.

The difference in growth recovery between partially and fully saturated hLf could be due to an increase in the quantity of Lf-bound iron. Insufficient iron, however, does not account for the decrease in growth seen with partially saturated hLf. It could be that *H. pylori* is able to access the iron bound to holo-Lf more efficiently. The mechanism by which *H. pylori* acquires iron from Lf is unknown, but may include an LBP, flavin reductases, or other mechanisms.

Previous work has suggested that *H. pylori* expresses an LBP that specifically interacts with hLf (Dhaenens *et al.* 1997). Lf binding proteins are best characterized in the Neisseriaceae family. Interestingly, while all natural isolates of *Neisseria meningitidis* make functional Lf receptors, only half of *Neisseria gonorrhoeae* do (Anderson *et al*. 2003). Similarly, though *N. meningitidis* invariably expresses Lf receptors, a number of bacterial species that inhabit the same nasopharyngeal niche do not (Ling and Schryvers 2006). This suggests that while Lf *can* be an iron-source in these conditions, it does not have to be. Ling and Schryvers suggested that there is evidence for opposing selective forces for LBP expression in different niches that may be separate from iron-binding, and, while iron-acquisition appears to be an important role of LBPs in some bacteria, this does not preclude LBPs having additional functions *in vivo*.

The presence of an LBP may affect the ability of hLf to adhere to and invade epithelial cells. Lf is bound and internalized by receptors on epithelial cell surfaces, and by binding hLf bound to cell surfaces, *H. pylori* may be able to adhere to and invade at a higher frequency.

Here, *H. pylori* was found to adhere to epithelial cells at a higher frequency with both bLf and hLf, irrespective of the level of iron in the medium. This may be due to the ability of bLf and hLf to non-specifically bind components of bacterial and epithelial cell surfaces. Internalization was increased to a similar degree with bLf in both conditions, and hLf in

iron-replete conditions, suggesting that an increase in adhesion is related to an increase in internalization in these situations.

Interestingly, internalization with hLf in iron-limiting conditions was 18-fold higher than the iron-limited control, far higher than any other condition. Adherence was not increased to the same degree as internalization, suggesting that a larger proportion of total adherent bacteria are being internalized under these conditions. The increased internalization seen in iron-limiting conditions could be due to an iron-regulated increase in the production of cellular receptors for binding and internalizing Lf, known to occur in some cell lines (Mikogami *et al.* 1995, McAbee and Ling 1997, Olakanmi *et al.* 2002). *H. pylori* could be binding the Lf bound to cell receptors via an LBP, also believed to be upregulated in iron-limiting conditions, or through non-specific binding of hLf to the bacterial outer membrane. This could be confirmed with microscopy work, to see if *H. pylori* and Lf co-localize on cell surfaces, possibly in relation to cell or bacterial receptors for Lf.

Lf has been shown to affect the internalization of a number of facultative intracellular bacteria, usually decreasing it by blocking binding sites on cell surfaces or degrading necessary bacterial proteins (Ajello *et al.* 2002, Di Biase *et al.* 2004, Superti *et al.* 2004). Should the increase in internalization by bLf and especially hLf seen here be confirmed, the effect of Lf would be markedly different from what has been seen using other species.

Lf was also investigated in relationship to its role as a DNA vector or transcription factor. Lf has been shown to bind sequences of DNA with a high affinity, and increase the transcription of downstream eukaryotic genes both *in vitro* and *in vivo* (He and Furmanksi 1995, Son *et al.* 2002, Mariller *et al.* 2007). The possibility exists that Lf may also be able to act as a transcription factor in prokaryotes. Bioinformatics tools were used to determine if Lf binding sites occur within 100 bacterial genomes, if they

were more or less prevalent than expected, and, in the genome of *H. pylori*, if they occur in locations that would suggest a function in transcriptional regulation.

The number of Lf binding sites for each species was compared to the likelihood that a random sequence of the same length and base composition would occur in a particular genome. Though the majority of species had at least one sequence that was more or less abundant than expected, these incidences appeared to be randomly distributed across species.

As sequences do not need to be enriched in a genome to be functional, the location of individual binding sites in the genome of *H. pylori* in relation to downstream ORFs was also investigated. Only 2 of 70 Lf binding sites occurred near a promoter (within 100 bps upstream). Though elements appeared to be enriched in the interval between 200-400 bp away from the nearest downstream ORF, similar distributions were seen for random sequences of the same length and base composition.

No patterns emerged from this study that suggested that Lf binding sites were occurring in higher than expected numbers across species, or, in the case of *H. pylori*, in locations likely to be relevant to a transcription factor. Yet this does not preclude their use in individual species. Further work *in vitro* and *in vivo* would be needed to confirm or negate Lf's ability to act as a transcription factor in prokaryotes. For one, it is not yet known if bacteria regularly internalized Lf, which could be examined with labeled Lf. Additionally, bacteria could be transformed with Lf expression vectors and plasmids containing Lf binding sequences upstream of reporter genes, similar to the work first describing Lf-mediated upregulation of transcription in eukaryotes.

This study confirmed the presence of high affinity Lf-binding sites in all prokaryotic genomes surveyed. Lf interacts with both bacterial cells and mammalian cells, and could bind and shuttle DNA in the extracellular

environment, possibly even bringing DNA into human cells. Furthermore, Lf increases the chances of gene transfer within the intracellular environment by increasing the uptake of *H. pylori* into epithelial cells.

Future Work

Much of the work done here can be used to chart future confirmatory experiments. One way to expand on this research would be to determine if there are strain-specific effects of Lf, effects that might differ between laboratory strains and clinical isolates. Previous work has shown that strains react differently to iron-limitation, including storage of intracellular iron, levels of growth in low-iron environments and changes in outer membrane profiles (Bland *et al.* 2004, Lee *et al.* 2009).

H. pylori exhibits a high degree of inter- and intra-strain variability, and significant genomic changes can occur after a relatively small number of laboratory passages as well as from strains within the same host over time (Kuipers *et al.* 2000, Bourzac and Guillemin 2005, Baltrus *et al.* 2007). All three strains, 60190, Tx30a, and SS1 are common laboratory strains and extensive passaging may have changed their interactions with hLf. Testing clinical isolates associated with different virulence phenotypes could shed light on how Lf interactions affect bacterial pathogenesis.

The ability to use hLf as an iron-source could give *H. pylori* strains a selective advantage in the stomach, and may increase their pathogenicity. Patients with *H. pylori*-related, iron-deficient anemia are colonized with strains that produce more IROMPs and are more efficient users of iron (Lee *et al.* 2009). Some of these IROMPs may be involved in sequestering iron from hLf. Severe gastritis has been associated both with an increase in intracellular *H. pylori in vivo*, and higher levels of hLf in the gut (Chan

1992, Wen *et al.* 2004). Furthermore, intracellular bacteria may be able to evade antibiotic treatment, and thus persist for longer (Heinemann 1999, Peterson and Krogfelt 2003).

A good deal of information on how *H. pylori* is interacting with Lf would depend on isolation and characterization of the putative LBP. While the effects seen here are consistent with the presence of an LBP, they could be mediated by other factors. Isolation of an LBP and the LBP gene would allow for investigation of the mechanisms by which *H. pylori* uses Lf-bound iron and invades epithelial cells in the presence of Lf. Similar studies could also be performed with other species of bacteria known to use hLf and invade epithelial cells, such as *Neisseria* sp. The LBP of *Neisseria* sp is well characterized, and work could be done on isogenic strains to see if the affects on internalization were LBP mediated. It would also serve as a point of comparison to how other Lf-adapted bacteria interact with Lf.

The bioinformatics work done here suggested that Lf binding sites exist in a wide range of bacteria. If time had permitted, Lf would have been tested for its potential to act as a vector for DNA uptake by *H. pylori* by measuring rates of transformation in the presence or absence of plasmid-bound Lf. Standard plasmids, with selective marker genes, would be modified with putative Lf binding sequences and used to visualize and select for the transfer of genetic material. During the course of this investigation, an application was prepared and filed with ERMA NZ to investigate the movement of plasmids containing Lf-binding sites from bacteria to epithelial cells, yeast cells, phage, and between ten species of gut and soil bacteria. This will allow continuing work to be done on Lf's potential to act as a DNA vector for both prokaryotes and eukaryotes.

Interactions between bacteria and Lf are important in light of the current interest in utilizing Lf's wide range of anti-microbial activities for commercial and therapeutic purposes. A number of plants and animals

have been investigated as potential biofactories for the mass production of recombinant hLf (Heinemann 2008). The mass production of hLf in non-human hosts will both increase the range of environments, numbers and kinds of bacteria that will be exposed to Lf, and increase the quantity of Lf they will be exposed to.

Bacteria that live in high Lf environments have adapted to its presence, ranging from tolerance of the normally anti-microbial peptide to use of it as a source of iron and possibly for other purposes (Ling and Schryvers 2006). While resistance to anti-microbial peptides is reportedly rare, commercial use and the associated increase in bacterial exposure to Lf could greatly amplify the selective pressure for resistance among bacteria not already tolerant (Heinemann 2008). Already, it has been found that resistance to bovine Lfcin is rapidly induced in *Staphylococcus aureus* in the laboratory, and has been reported in clinical isolates of *S. aureus* small colony variants (Samuelson *et al.* 2005a,b). Because hLf is part of the human immune system, widespread resistance to its antimicrobial properties could compromise our natural defense against infection (Bell and Gouyon 2003).

Depending on how the bacteria became resistant to Lf, this resistance could potentially spread to other species via horizontal gene transfer. Issues of horizontal gene transfer are especially pertinent for risk assessment of genetically modified organisms, involving the spread of resistance as well as the movement of transgenes into other, non-GMO species. Because Lf can act as DNA vector, the presence of Lf binding sites within bacterial DNA suggests that it might increase horizontal gene transfer not just within its natural host but also within new organisms and new environments. Lf could bind DNA in animals and possibly plants *in vivo*, and shuttle it to microbes in and on transgenic plants and animals used as biofactories (Heinemann 2008).

Our ability to assess the risks of undertakings such as biopharming, the use of GMOs to produce pharmaceuticals, first relies on a solid understanding of Lf's interactions with prokaryotes: those that are adapted to its presence and those that might become adapted to it.

Appendix I
Additional Material and Methods

Chapter 2

Media

Brucella Broth Solution

(per liter)

Pancreatic digest of casein	10 g
Peptic digest of animal tissue	10 g
Dextrose	1.0 g
Yeast extract	2.0 g
Sodium Chloride	5.0 g
Sodium bisulfite	0.1 g

Columbia Blood Agar Plates

(per liter)

Special peptone	23.0 g
Starch	1.0 g
Sodium Chloride	5.0 g
Agar No. 1	39.0 g
Defibrinated sheep blood	50.0 ml

(Prepared by Fort Richard, NZ)

Protein Assay Solutions

Solution A (w/v)

Sodium carbonate	2 %
Sodium hydroxide	0.4 %
Potassium sodium-tartrate	0.16 %
Sodium dodecyl sulphate (SDS)	1 %

in dH_20

Solution B (w/v)

Copper sulphate crystals	0.5 %

in dH_20

Solution C

Solution A	50 ml
Solution B	1 ml

SDS-PAGE Solutions

Resolving Gel (12.5 %)

40 % Acrylamide/Bis solution (BioRad)	3.13 ml
Resolving Gel Buffer (below)	2.5 ml
dH2O	4.37 ml
10 % Ammonia Persulphate (APS)	100 µl
NNN'N'-tetramethylethylene (TEMED)	20 µl

Stacking Gel (4.5 %)

40 % Acrylamide/Bis solution (BioRad)	450 µl
Stacking Gel Buffer (below)	1 ml
dH2O	2.55 ml
APS	100 µl
TEMED	20 µl

Resolving Gel Buffer (w/v)

Tris	18.3 %
SDS	0.4 %

in dH_20

Stacking Gel Buffer (w/v)

Tris	6.06 %
SDS	0.4 %

in dH_20

Reservoir Buffer (1.8 liters)

Tris	5.4 g
Glycine	25.92 g
SDS	1.8 g
dH_20	1.76 l

Silver Staining Solutions

Farmers Reducer (w/v)

Potassium ferricyanide	0.15 g
Sodium thiosulphate	0.3 %
Sodium carbonate	0.05 %

in dH_20

Chapter 3

N-P40 solutions

Tris-HCl (7.5 pH)	50mM
NaCl	100mM
nanodine P40	1 %

Appendix II
Raw and Supplemental Data

Chapter 2

Table A2.1: Effect of increasing iron-limitation on the growth of *H. pylori* strain 60190.

	14-Aug	16-Aug	28-Sep	Mean	STDEV	STERR
IR Control	100	100	100	100	0	0
10uM DE	43	14	105	54	47	27
20uM DE	4	15	4	8	6	4
35uM DE	4	21	2	9	11	6
50uM DE	2	29	2	11	15	9

Table A2.2: Iron supplementation (10 μM) rescues growth of *H. pylori* strain 60190 in iron-limiting conditions

	14-Aug	16-Aug	28-Sep	Mean	STDEV	STERR
IR Control	100	100	100	100	0	0
10uM DE	84	124	111	106	20	12
20uM DE	90	134	108	111	22	13
35uM DE	37	136	107	93	51	29
50uM DE	2	48	5	19	26	15

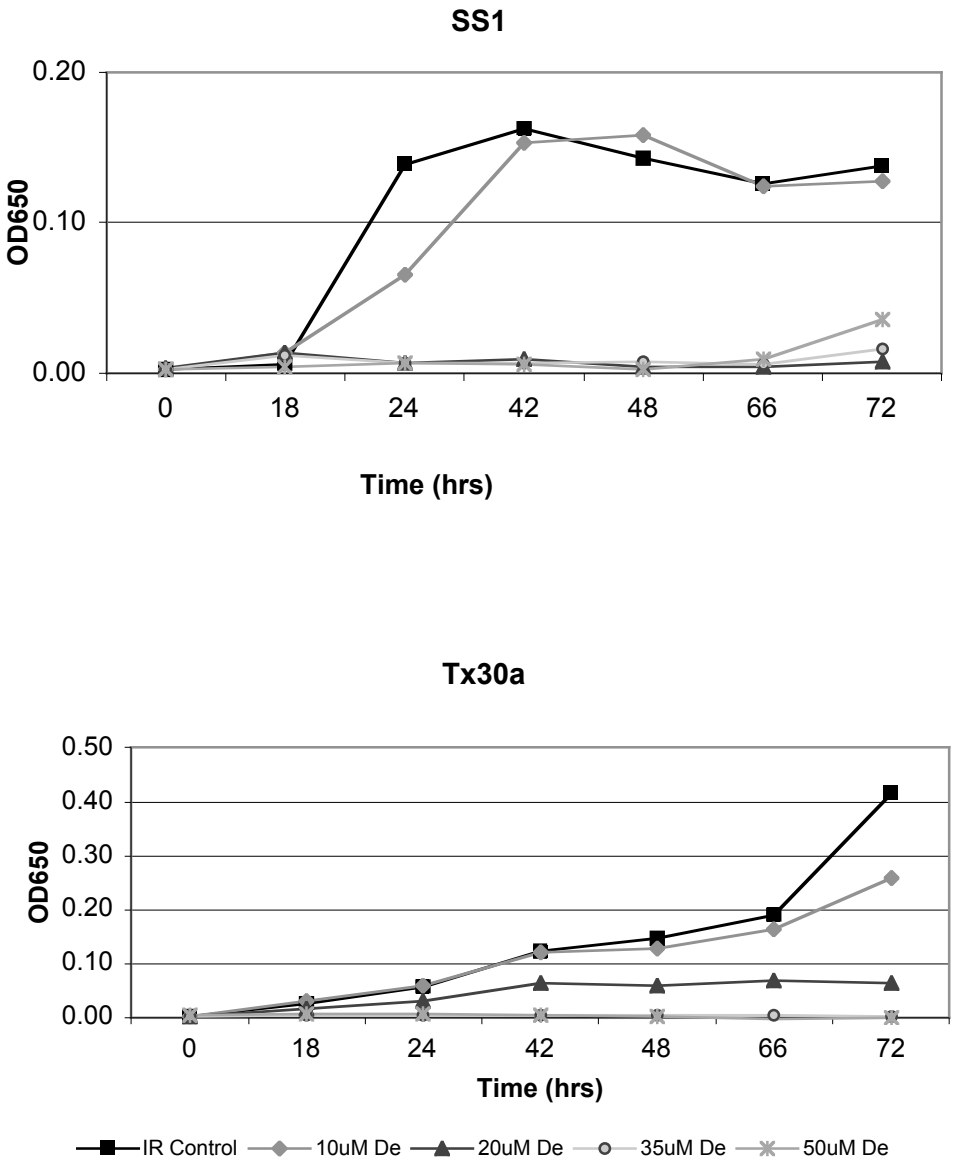

Figure A2.1: Effect of increasing levels of iron-limitation on the growth of *H. pylori* strains SS1 and Tx30a. Bacteria were grown in iron-replete media (IR control) and iron-limited media (with10-50 μM DE) supplemented with 10 μM $FeCl_3$ to determine if it was sufficient iron for recovery of growth. Growth was measured over 72 hrs from one representative trial

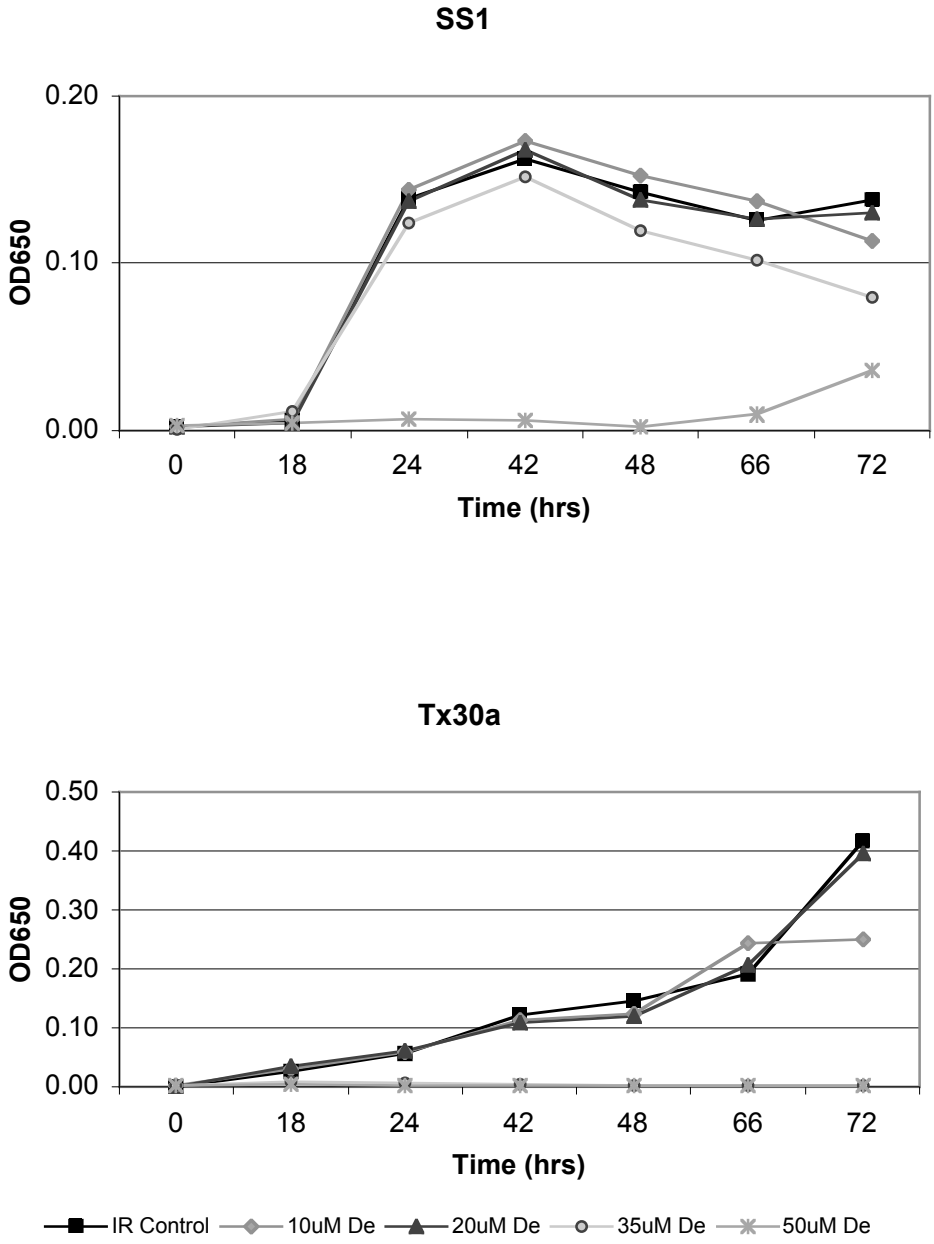

Figure A2.2: Growth recovery of *H. pylori* strains SS1 and Tx30a with iron supplementation in iron-limiting conditions. Bacteria were grown in iron replete media (IR control) and iron-limited media (with10–50 μM DE) supplemented with 10 μM $FeCl_3$ to determine if it was sufficient iron for recovery of growth**.** Growth was measured over 72 hrs in one representative trial.

Table A2.3: Growth of strain 60190 with 0.5 mg/ml of partially saturated human lactoferrin.

	24-Sep	4-Nov	7-Nov (1)	7-Nov (2)	Mean	STDEV	STERR
IR Control	100	100	100	100	100.0	0.0	0.0
IL Control	6	4	29		11.4	10.7	4.8
IR + hLf		62	106	78	82.0	22.3	12.9
IL + hLf	8	0	3	3	3.5	3.3	1.7
IL + FeCl3	116	108		82	106.0	20.7	9.3

Table A2.4: Growth of strain SS1 with 0.5 mg/ml of partially iron-saturated human lactoferrin, with growth inhibition in iron-replete conditions.

	28-Oct	4-Nov	7 Nov (1)	7 Nov (2)	Mean	STDEV	STERR
IR Control	100	100	100	100	100.0	0.0	0.0
IL Control	3	25	2		10.0	13.0	7.5
IR + hLf		82	84	82	82.7	1.2	0.7
IL + hLf		1	2	2	1.7	0.6	0.3
IL + FeCl3	97		86	93	92.0	5.6	3.2

Table A2.5: Growth of strain Tx30a with 0.5 mg/ml of partially iron-saturated human lactoferrin, with growth inhibition in iron-limiting conditions

	4 Nov (1)	4 Nov (2)	7 Nov (1)	Mean	STDEV	STERR
IR control	100	100	100	100.0	0.0	0
IL control	40	41	95	58.7	31.5	18.2
IR+hLf	103	95	109	102.3	7.0	4.1
IL+hLf	1	1	45	15.7	25.4	14.7
IL+FeCl3	82		86	84.0	2.8	1.6

Table A2.6: Possible growth inhibition of strain 60190 with 1 mg/ml of partially iron-saturated human lactoferrin.

	9-Dec	12-Dec	AVE	STDEV	STERR
IR Control	100	100	100.0	0.0	0.0
IL Control	1	1	1.3	0.0	0.0
IR+hLf	80	63	71.8	11.9	6.9
IL+hLf	1	1	1.3	0.1	0.1
IL+FeCl3	92	80	86.2	8.3	4.8

Table A2.7: Growth recovery of strain 60190 in iron-limiting conditions with 0.5 mg/ml of fully iron-saturated human lactoferrin.

	10-Feb	11-Feb	12-Feb	Mean	STDEV	STERR
IR Control	100	100	100	99.9	0.1	0.0
IL Control	24	40	37	33.5	8.1	4.7
IR+hLf	142	131	107	126.5	18.1	10.4
IL+hLf	103	171	89	120.9	44.1	25.4
IL+FeCl3	85	89	78	84.1	6.0	3.5

Chapter 3

Table A3.1: **Frequency of Internalization into Epithelial cells of *H. pylori* strain 60190 with hLf in FBS-mediated iron-limiting conditions.**

	12-Sep	6-Oct	21-Oct	Mean	STDEV	STERR
Total Bacteria Recovered						
(-) FCS	1428	300	18500	6743	10198	5888
(+)FCS	15675	3050	149000	55908	80867	46688
(-)FCS(+)LF	16275	200	5300	7258	8214	4743
(+)FCS(+)LF	9925	3125	6350	6467	3402	1964
Percentage Internalization of Innoculum						
(-) FCS	0.002	0.007	0.235	0.081	0.133	0.077
(+)FCS	0.021	0.067	1.900	0.662	1.072	0.619
(-)FCS(+)LF	0.022	0.004	0.068	0.031	0.033	0.019
(+)FCS(+)LF	0.013	0.068	0.081	0.054	0.036	0.021

Table A3.2: Frequency of Internalization into Epithelial cells of *H. pylori* strain 60190 with Lf in DE-mediated iron-limiting conditions.

	30-Oct	3-Nov	7-Nov	11-Dec	Mean	STDEV	STERR
Total Recovered Bacteria							
(-)DE	5800	3000	2325	16200	6831	6424	3212
(+)DE	550	300	775	9170	2699	4319	2159
(-)DE (+)bLF	16400	20600	1075	16700	13694	8627	4314
(+)DE (+)bLF	2330	4750	1500	16400	6245	6909	3454
(-)DE (+)hLF	12900	14500	825	36400	16156	14812	7406
(+)DE (+)hLF	4250	26500	19300	20300	17588	9445	4722
Percent Internalization of Innoculum							
(-)DE	0.091	0.061	0.062	0.007	0.055	0.035	0.017
(+)DE	0.009	0.006	0.021	0.004	0.010	0.007	0.004
(-)DE (+)bLF	0.257	0.417	0.029	0.008	0.178	0.196	0.098
(+)DE (+)bLF	0.037	0.096	0.039	0.007	0.045	0.037	0.019
(-)DE (+)hLF	0.203	0.294	0.022	0.166	0.171	0.113	0.057
(+)DE (+)hLF	0.067	0.535	0.513	0.009	0.281	0.282	0.141

Table A3.3: Frequency of Internalization into Epithelial cells of *H. pylori* strain 60190 with denatured Lf in DE-mediated iron-limiting conditions.

	21-Jan	27-Jan	28-Jan	Mean	STDEV	STERR
Total Bacteria Recovered						
(-)DE	22600	10005	21300	17968	6927	3999
(+)DE	18500	2900	6900	9433	8103	4678
(-)DE(+)dhLf	12800	8500	8850	10050	2388	1379
(+)DE(+)dhLf	9950	2850	4300	5700	3751	2166
Percent Internalization of Inoculum						
(-)DE	0.995	2.080	4.750	2.608	1.932	1.116
(+)DE	0.811	0.604	1.540	0.985	0.492	0.284
(-)DE(+)dhLf	0.561	1.770	1.975	1.435	0.764	0.441
(+)DE(+)dhLf	0.436	0.580	0.959	0.658	0.270	0.156

Table A3.4: Frequency of Adhesion onto Epithelial cells of *H. pylori* strain 60190 with Lf in DE-mediated iron-limiting conditions.

	28-Nov	1 Dec (1)	1 Dec (2)	Mean	STDEV	STERR
Total Bacteria Recovered						
(-)DE	35200	49500	502000	100	0	0
(+)DE	10200	10500	205000	30	10	6
(-)DE(+)bLF	35000	620000	923000	512	643	371
(+)DE(+)bLF	22400	40100	2820000	235	283	163
(-)DE(+)hLF	122000	104000	1770000	303	81	47
(+)DE(+)hLF	42100	43300	1280000	154	89	51
Percent Adhesion of Innoculum						
(-)DE	0.567	0.481	6.920	2.656	3.693	2.132
(+)DE	0.164	0.102	2.820	1.029	1.552	0.896
(-)DE(+)bLF	0.564	6.020	12.700	6.428	6.078	3.509
(+)DE(+)bLF	0.361	0.389	38.900	13.217	22.242	12.842
(-)DE(+)hLF	1.970	1.010	24.400	9.127	13.236	7.642
(+)DE(+)hLF	0.678	0.420	17.700	6.266	9.903	5.717

Chapter 4

Table A4.1: Occurance of LFREs Across Genomes. Genomes were searched for the presence of five LFREs. The total number of LFREs was calculated and divided by the genome size to get number of LFREs per kilo-base-pair.

	LFREs						
Species	1a	1b	2a	2b	3	Total	per kbp
Actinobacteria-Actinomycetales							
Corynebacterium diphtheriae NCTC 13129	72	217	0	12	0	301	0.121
Corynebacterium efficiens YS-314	133	191	2	5	0	331	0.105
Mycobacterium bovis AF2122/97	181	285	1	1	0	468	0.108
Mycobacterium tuberculosis CDC1551	179	292	1	1	0	473	0.107
Streptomyces avermitilis MA-4680	369	906	0	2	0	1277	0.141
Streptomyces coelicolor A3(2)	410	863	0	0	0	1273	0.147
Alpha Proteobacteria							
Agrobacterium tumefaciens str. C58	320	352	5	18	0	695	0.141
Bradyrhizobium japonicum USDA 110	247	558	3	5	0	813	0.089
Brucella abortus biovar 1 str. 9-941	84	217	0	4	0	305	0.093
Brucella melitenus 16M	83	216	0	4	0	303	0.092
Ehrlichia canis str. Jake	6	44	12	9	0	71	0.054
Ehrlichia chaffeensis str. Arkansas	6	55	5	4	0	70	0.060
Ehrlichia ruminantium str. Gardel	5	60	2	4	0	71	0.047
Nitrobacter hamburgensis X14	133	273	0	1	0	407	0.092
Rhizobium leguminosarum bv. viciae 3841	177	453	6	5	0	641	0.127
Rhodospirillum rubrum	91	67	2	1	0	161	0.037

Rickettsia akari str. Hartford	13	57	4	2	0	76	0.062
Rickettsia rickettsii	9	76	4	2	0	91	0.072
Aquificae							
Aquifex aeolicus VF5	10	137	2	4	0	153	0.099
Bacteriodetes							
Bacteroides fragilis NCTC 9343	95	523	6	20	0	644	0.124
Bacteroides fragilis YCH46	97	530	5	25	0	657	0.125
Bacteroides thetaiotaomicron VPI-5482	143	701	4	18	0	866	0.138
Cytophaga hutchinsonii ATCC 33406	89	330	2	7	0	428	0.097
Flavobacterium psychrophilum JIP02/86	49	121	5	3	0	178	0.062
Bacteroides fragilis NCTC 9343	95	523	6	20	0	644	0.124
Bacteroides fragilis YCH46	97	530	5	25	0	657	0.125
Bacteroides thetaiotaomicron VPI-5482	143	701	4	18	0	866	0.138
Cytophaga hutchinsonii ATCC 33406	89	330	2	7	0	428	0.097
Flavobacterium psychrophilum JIP02/86	49	121	5	3	0	178	0.062
Beta Proteobacteria							
Bordetella bronchiseptica RB50	216	368	1	1	0	586	0.110
Bordetella parapertussis 12822	202	352	2	0	0	556	0.116
Bordetella pertussis Tohama I	149	261	1	4	0	415	0.102
Burkholderia ambifaria AMMD	260	945	1	1	0	1207	0.157
Burkholderia cenocepacia AU 1054	259	910	2	3	0	1174	0.161
Chromobacterium violaceum ATCC 12472	90	204	3	1	0	298	0.063
Neisseria gonorrhoeae FA 1090	50	147	0	1	0	198	0.092

Neisseria meningitidis FAM18	53	162	0	2	0	217	0.099
Nitrosomonas europaea ATCC 19718	67	300	0	2	0	369	0.131
Nitrosomonas eutropha C71	63	251	3	8	0	325	0.122
Cyanobacteria							
Nostoc sp. PCC 7120	141	386	11	36	0	574	0.070
Prochlorococcus marinus str. MIT 9211	14	100	12	9	0	135	0.080
Synechococcus elongatus PCC 6301	49	140	2	12	0	203	0.075
Synechococcus sp. CC9605	79	116	1	8	0	204	0.078
Deinococcus-Thermus							
Deinococcus geothermalis DSM11300	162	263	0	6	0	431	0.175
Deinococcus radiodurans R1	207	303	0	8	0	518	0.169
Thermus thermophilus HB27	33	70	0	5	0	108	0.057
Thermus thermophilus HB8	37	70	0	6	0	113	0.061
Delta Proteobacteria							
Bdellovibrio bacteriovorus HD100	87	286	0	3	0	376	0.099
Desulfovibrio desulfuricans G20	0	280	0	0	0	280	0.075
Desulfovibrio vulgaris subsp. vulgaris str. Hildenborough	227	474	0	5	0	706	0.197
Desulfuromonas acetoxidans	200	380	3	0	0	583	0.165
Geobacter metallireducens GS-15	129	266	3	4	0	402	0.101
Pelobacter carbinolicus DSM 2380	91	217	2	5	0	315	0.086
Syntrophobacter fumaroxidans MPOB	131	359	1	5	0	496	0.099
Epsilon Proteobacteria							
Campylobacter jejuni subsp. doylei 269.97	3	32	8	15	0	58	0.031
Campylobacter jejuni subsp. jejuni 260.94	4	34	7	9	0	54	0.033

Campylobacter jejuni subsp. jejuni 81-176	6	38	5	10	0	59	0.037
Helicobacter pylori 26656	27	37	8	2	0	74	0.044
Helicobacter pylori J99	1	42	8	19	0	70	0.043
Thiomicrospira crunogena XCL-2	32	124	5	8	0	169	0.070
Wolinella succinogenes DSM 1740	11	39	1	15	0	66	0.031
Firmicutes Bacilli							
Bacillus anthracis str. A1055	95	415	14	9	0	533	0.101
Bacillus anthracis str. 'Ames Ancestor'	95	424	14	9	0	542	0.104
Bacillus cereus ATCC 10987	81	439	18	8	0	546	0.105
Bacillus cereus ZK	83	412	12	10	0	517	0.098
Bacillus licheniformis ATCC 14580	54	169	2	9	0	234	0.055
Bacillus thuringiensis serovar konkukian str. 97-27	87	408	14	6	0	515	0.098
Enterococcus faecalis V583	38	193	3	19	0	253	0.079
Lactobacillus acidophilus NCFM	0	152	0	0	0	152	0.076
Lactobacillus brevis ATCC 367	0	266	0	0	0	266	0.116
Lactobacillus gasseri ATCC 33323	0	126	0	0	0	126	0.066
Lactococcus lactis subsp. lactis Il1403	17	136	2	11	0	166	0.070
Staphylococcus aureus RF122	27	155	3	10	0	195	0.078
Staphylococcus haemolyticus JCSC1435	24	137	8	11	0	180	0.067
Streptococcus pneumoniae 23F	20	148	2	16	0	186	0.085
Streptococcus uberis 0140J	18	112	4	14	0	148	0.087
Firmicutes Clostridium/Mollicutes							
Clostridium acetobutylicum ATCC	28	174	2	15	0	219	0.056

824							
Clostridium botulinum A str. ATCC 19397	14	99	24	12	0	149	0.039
Spiroplasma kunkelii CR2-3x	1	96	2	3	0	102	0.064
Gamma Proteobacteria							
Acinetobacter baumannii ATCC 17978	68	330	6	17	0	421	0.106
Azotobacter vinelandii	143	277	0	4	0	424	0.094
Escherichia coli K12	140	349	2	3	0	494	0.106
Escherichia coli O157:H7	183	404	0	1	0	588	0.095
Francisella tularensis subsp. holarctica FTA	6	72	4	6	0	88	0.047
Francisella tularensis subsp. tularensis FSC198	6	71	4	6	0	87	0.046
Haemophilus influenzae 86-028NP	3	136	3	18	0	160	0.084
Legionella pneumophila str. Lens	38	174	5	10	0	227	0.068
Methylococcus capsulatus str. Bath	125	166	0	2	0	293	0.089
Photobacterium profundum 3TCK	124	517	7	11	0	659	0.108
Pseudomonas aeruginosa 2192	181	354	1	3	0	539	0.079
Pseudomonas aeruginosa PAO1	165	312	1	1	0	479	0.076
Pseudomonas aeruginosa UCBPP-PA14	163	340	1	1	0	505	0.077
Salmonella bongori 12149	126	320	3	4	0	453	0.103
Salmonella enterica subsp. enterica serovar Choleraesuis str. SC-B67	125	308	0	3	0	436	0.092
Salmonella typhimurium LT2	127	302	0	0	0	429	0.088
Vibrio alginolyticus 12G01	123	555	9	18	0	705	0.137
Vibrio cholerae AM-19226	100	324	2	9	0	435	0.107
Vibrio splendidus 12B01	0	479	0	0	0	479	0.086
Vibrio vulnificus YJ016	139	477	2	10	0	628	0.121
Planctomycetes							
Pirellula sp. 1	159	621	2	8	0	790	0.111

Spirochaetes							
Borrelia afzelii PKo	2	36	2	4	0	44	0.049
Borrelia burgdorferi B31	6	28	2	12	0	48	0.053
Leptospira interrogans serovar Copenhageni str. Fiocruz L1-130	19	212	11	20	0	262	0.061
Treponema denticola ATCC 35405	29	124	1	5	0	159	0.056
Treponema pallidum subsp. pallidum str. Nichols	50	195	0	2	0	247	0.217
Thermotogae							
Thermotoga maritima MSB8	29	133	4	7	0	173	0.093
Thermotoga petrophila RKU-1	27	120	3	5	0	155	0.083

Table A4.2: Occurrence of lactoferrin binding sites in comparison to expected frequencies[1] by LFRE consensus sequence

LFRE1a				
	Mean Occ. of Random LFREs	95 % CI	Obs. Occ. of Actual LFREs	+/-
Actinobacteria-Actinomycetales				
Corynebacterium diphtheriae NCTC 13129	83.90	15.01	72	
Corynebacterium efficiens YS-314	91.60	34.63	133	+
Mycobacterium bovis AF2122/97	127.80	45.85	181	+
Mycobacterium tuberculosis CDC1551	130.00	46.75	179	+
Streptomyces avermitilis MA-4680	299.00	179.4	369	
Streptomyces coelicolor A3(2)	299.60	186.1	410	
Alpha Proteobacteria				
Agrobacterium tumefaciens str. C58	142.00	69.19	320	+
Bradyrhizobium japonicum USDA 110	271.20	169.4	247	
Brucella abortus biovar 1 str. 9-941	62.80	26.76	84	
Brucella melitenus 16M	63.50	27.05	83	

Ehrlichia canis str. Jake	9.10	3.94	6	
Ehrlichia chaffeensis str. Arkansas	10.20	4.03	6	
Ehrlichia ruminantium str. Gardel	10.50	3.91	5	-
Nitrobacter hamburgensis X14	143.20	78.53	133	
Rhizobium leguminosarum bv. viciae 3841	206.00	127.4	177	
Rhodospirillum rubrum	116.40	63.67	91	
Rickettsia akari str. Hartford	11.10	6.77	13	
Rickettsia rickettsii	12.20	5.34	9	
Aquificae				
Aquifex aeolicus VF5	41.60	30.43	10	-
Bacteriodetes				
Bacteroides fragilis NCTC 9343	83.70	17.14	95	
Bacteroides fragilis YCH46	85.90	16.29	97	
Bacteroides thetaiotaomicron VPI-5482	103.60	22.68	143	+
Cytophaga hutchinsonii ATCC 33406	52.70	13.67	89	+
Flavobacterium psychrophilum JIP02/86	23.80	6.39	49	+
Beta Proteobacteria				
Bordetella bronchiseptica RB50	141.90	69.69	216	
Bordetella parapertussis 12822	127.40	59.44	202	+
Bordetella pertussis Tohama I	123.1	80.67	149	
Burkholderia ambifaria AMMD	197.90	150.5	260	
Burkholderia cenocepacia AU 1054	194.60	158.2	259	
Chromobacterium violaceum ATCC 12472	140.40	83.51	90	
Neisseria gonorrhoeae FA 1090	33.20	15.69	50	+
Neisseria meningitidis FAM18	38.80	18.80	53	
Nitrosomonas europaea ATCC 19718	64.50	20.39	67	
Nitrosomonas eutropha C71	62.40	22.67	63	
Cyanobacteria				
Nostoc sp. PCC 7120	105.80	33.73	141	+
Prochlorococcus marinus str. MIT 9211	32.20	9.90	14	+
Synechococcus elongatus PCC 6301	90.80	25.22	49	-
Synechococcus sp. CC9605	84.90	32.11	79	
Deinococcus-Thermus				
Deinococcus geothermalis	92.70	30.47	162	+

DSM11300				
Deinococcus radiodurans R1	87.30	32.47	207	+
Thermus thermophilus HB27	111.80	78.68	33	
Thermus thermophilus HB8	109.70	74.43	37	
Delta Proteobacteria				
Bdellovibrio bacteriovorus HD100	92.90	33.43	87	
Desulfovibrio desulfuricans G20	89.10	29.38		
Desulfovibrio vulgaris subsp. vulgaris str. Hildenborough	114.10	43.67	227	+
Desulfuromonas acetoxidans	137.60	60.02	129	
Geobacter metallireducens GS-15	81.00	22.28	91	
Pelobacter carbinolicus DSM 2380	160.90	87.21	131	
Syntrophobacter fumaroxidans MPOB	92.90	33.43	87	
Epsilon Proteobacteria				
Campylobacter jejuni subsp. doylei 269.97	13.00	3.97	3	-
Campylobacter jejuni subsp. jejuni 260.94	12.30	5.82	4	-
Campylobacter jejuni subsp. jejuni 81-176	12.60	6.25	6	-
Helicobacter pylori 26656	19.4	9.04	27	
Helicobacter pylori J99	20.80	8.77	1	-
Thiomicrospira crunogena XCL-2	39.80	14.32	32	
Wolinella succinogenes DSM 1740	42.70	13.48	11	-
Firmicutes Bacilli				
Bacillus anthracis str. A1055	57.00	7.80	95	+
Bacillus anthracis str. 'Ames Ancestor'	58.20	8.13	95	+
Bacillus cereus ATCC 10987	61.80	7.56	81	+
Bacillus cereus ZK	57.70	8.09	83	+
Bacillus licheniformis ATCC 14580	80.40	35.56	54	
Bacillus thuringiensis serovar konkukian str. 97-27	60.10	11.21	87	+
Enterococcus faecalis V583	44.80	11.86	38	
Lactobacillus acidophilus NCFM	30.80	8.24	0	-
Lactobacillus brevis ATCC 367	27.80	10.34	17	
Lactobacillus gasseri ATCC 33323	23.60	5.15	27	
Lactococcus lactis subsp. lactis Il1403	25.60	6.24	24	
Staphylococcus aureus RF122	39.00	10.04	20	-
Staphylococcus haemolyticus	28.40	12.47	18	

JCSC1435				
Streptococcus pneumoniae 23F	35.90	15.90	28	
Streptococcus uberis 0140J	31.10	17.09	14	-
Firmicutes Clostridium/Mollicutes				
Clostridium acetobutylicum ATCC 824	35.90	15.90	28	
Clostridium botulinum A str. ATCC 19397	31.10	17.09	14	-
Spiroplasma kunkelii CR2-3x	6.50	2.68	1	-
Gamma Proteobacteria				
Acinetobacter baumannii ATCC 17978	54.20	12.61	68	
Azotobacter vinelandii	165.30	69.78	143	
Escherichia coli K12	97.30	36.84	140	+
Escherichia coli O157:H7	118.40	43.44	183	+
Francisella tularensis subsp. holarctica FTA	18.00	6.95	6	+
Francisella tularensis subsp. tularensis FSC198	23.80	12.52	6	+
Haemophilus influenzae 86-028NP	26.5	11.51	3	-
Legionella pneumophila str. Lens	54.20	18.88	38	
Methylococcus capsulatus str. Bath	111.40	58.10	125	
Photobacterium profundum 3TCK	106.30	22.35	124	
Pseudomonas aeruginosa 2192	207.50	86.06	181	
Pseudomonas aeruginosa PAO1	188.10	77.89	165	
Pseudomonas aeruginosa UCBPP-PA14	198.70	81.49	163	
Salmonella bongori 12149	112.10	39.61	126	
Salmonella enterica subsp. enterica serovar Choleraesuis str. SC-B67	120.50	43.74	125	
Salmonella typhimurium LT2	121.10	44.26	127	
Vibrio alginolyticus 12G01	106.30	23.31	123	
Vibrio cholerae AM-19226	91.30	26.70	100	
Vibrio splendidus 12B01	104.60	22.10		
Vibrio vulnificus YJ016	122.30	30.84	139	
Planctomycetes				
Pirellula sp. 1	205.50	84.00	159	
Spirochaetes				
Borrelia afzelii PKo	8.00	3.31	2	-
Borrelia burgdorferi B31	10.40	4.21	6	-
Leptospira interrogans serovar Copenhageni str. Fiocruz L1-130	48.60	19.96	19	-
Treponema denticola ATCC 35405	33.10	11.12	29	

Treponema pallidum subsp. pallidum str. Nichols	29.6	10.67	50	+
Thermotogae				
Thermotoga maritima MSB8	47.60	32.27	29	
Thermotoga petrophila RKU-1	48.30	31.64	27	
LFRE1b				
	Mean Occ. of Random LFREs	95 % CI	Obs. Occ. of Actual LFREs	+/-
Actinobacteria-Actinomycetales				
Corynebacterium diphtheriae NCTC 13129	259.00	58.90	217	
Corynebacterium efficiens YS-314	374.67	87.02	191	-
Mycobacterium bovis AF2122/97	529.33	169.8	285	-
Mycobacterium tuberculosis CDC1551	537.78	171.4	292	-
Streptomyces avermitilis MA-4680	797.33	113.6	906	
Streptomyces coelicolor A3(2)	780.67	116.4	863	
Alpha Proteobacteria				
Agrobacterium tumefaciens str. C58	611.67	173.9	352	-
Bradyrhizobium japonicum USDA 110	770.22	176.1	558	-
Brucella abortus biovar 1 str. 9-941	403.75	211.0	217	
Brucella melitenus 16M	417.89	200.2	216	-
Ehrlichia canis str. Jake	44.00	10.83	44	
Ehrlichia chaffeensis str. Arkansas	43.11	7.73	55	+
Ehrlichia ruminantium str. Gardel	45.22	11.99	60	+
Nitrobacter hamburgensis X14	560.44	171.5	273	-
Rhizobium leguminosarum bv. viciae 3841	749.56	173.1	453	-
Rhodospirillum rubrum	513.11	213.5	67	-
Rickettsia akari str. Hartford	62.78	20.87	57	
Rickettsia rickettsii	59.22	22.92	76	
Aquificae				
Aquifex aeolicus VF5	117.22	33.09	137	
Bacteriodetes				
Bacteroides fragilis NCTC 9343	443.33	81.60	523	
Bacteroides fragilis YCH46	449.22	76.85	530	+
Bacteroides thetaiotaomicron VPI-5482	498.67	95.86	701	+

Cytophaga hutchinsonii ATCC 33406	307.22	124.5	330	
Flavobacterium psychrophilum JIP02/86	144.11	46.93	121	
Beta Proteobacteria				
Bordetella bronchiseptica RB50	610.11	206.4	368	-
Bordetella parapertussis 12822	547.11	234.8	352	
Bordetella pertussis Tohama I	483.8	202.8	261	+
Burkholderia ambifaria AMMD	604.33	207.1	945	+
Burkholderia cenocepacia AU 1054	577.89	214.0	910	+
Chromobacterium violaceum ATCC 12472	592.11	211.2	204	-
Neisseria gonorrhoeae FA 1090	163.00	61.60	147	
Neisseria meningitidis FAM18	168.67	63.14	162	
Nitrosomonas europaea ATCC 19718	344.67	121.9	300	
Nitrosomonas eutropha C71	318.67	131.4	251	
Cyanobacteria				
Nostoc sp. PCC 7120	576.44	127.9	386	-
Prochlorococcus marinus str. MIT 9211	140.67	36.29	100	-
Synechococcus elongatus PCC 6301	352.56	129.1	140	-
Synechococcus sp. CC9605	337.67	109.4	116	-
Deinococcus-Thermus				
Deinococcus geothermalis DSM11300	356.11	88.58	263	-
Deinococcus radiodurans R1	356.89	85.86	303	
Thermus thermophilus HB27	224.78	85.40	70	-
Thermus thermophilus HB8	226.00	86.49	70	-
Delta Proteobacteria				
Bdellovibrio bacteriovorus HD100	388.44	128.2	286	
Desulfovibrio desulfuricans G20	449.00	157.0	280	-
Desulfovibrio vulgaris subsp. vulgaris str. Hildenborough	463.11	157.5	474	
Desulfuromonas acetoxidans	459.00	89.84	266	-
Geobacter metallireducens GS-15	443.89	138.9	217	-
Pelobacter carbinolicus DSM 2380	555.78	125.7	359	-
Syntrophobacter fumaroxidans MPOB	388.44	128.6	286	
Epsilon Proteobacteria				
Campylobacter jejuni subsp. doylei	75.33	27.35	32	-

269.97				
Campylobacter jejuni subsp. jejuni 260.94	54.00	21.07	34	
Campylobacter jejuni subsp. jejuni 81-176	58.63	18.93	38	-
Helicobacter pylori 26656	100.8	48.24	37	-
Helicobacter pylori J99	119.11	54.63	42	-
Thiomicrospira crunogena XCL-2	226.78	94.86	124	-
Wolinella succinogenes DSM 1740	199.44	56.20	39	-
Firmicutes Bacilli				
Bacillus anthracis str. A1055	313.00	143.0	415	
Bacillus anthracis str. 'Ames Ancestor'	317.11	142.9	424	
Bacillus cereus ATCC 10987	326.67	141.7	439	
Bacillus cereus ZK	315.22	144.5	412	
Bacillus licheniformis ATCC 14580	337.00	103.3	169	-
Bacillus thuringiensis serovar konkukian str. 97-27	314.67	140.3	408	
Enterococcus faecalis V583	244.67	114.1	193	
Lactobacillus acidophilus NCFM	112.78	39.80	152	
Lactobacillus brevis ATCC 367	142.00	38.39	136	
Lactobacillus gasseri ATCC 33323	151.33	63.83	155	
Lactococcus lactis subsp. lactis Il1403	141.22	41.22	137	
Staphylococcus aureus RF122	178.00	64.52	148	
Staphylococcus haemolyticus JCSC1435	114.67	23.39	112	
Streptococcus pneumoniae 23F	313.00	143.0	415	
Streptococcus uberis 0140J	317.11	142.9	424	
Firmicutes Clostridium/Mollicutes				
Clostridium acetobutylicum ATCC 824	166.11	50.11	174	
Clostridium botulinum A str. ATCC 19397	127.89	43.85	99	
Spiroplasma kunkelii CR2-3x	35.44	11.61	96	+
Gamma Proteobacteria				
Acinetobacter baumannii ATCC 17978	299.89	93.45	330	
Azotobacter vinelandii	679.56	175.7	277	-
Escherichia coli K12	504.78	204.7	349	
Escherichia coli O157:H7	559.89	191.5	404	

Francisella tularensis subsp. holarctica FTA	101.67	24.12	72	-
Francisella tularensis subsp. tularensis FSC198	103.44	23.58	71	-
Haemophilus influenzae 86-028NP	119.1	59.07	136	
Legionella pneumophila str. Lens	253.33	71.84	174	-
Methylococcus capsulatus str. Bath	461.67	135.9	166	-
Photobacterium profundum 3TCK	518.22	173.5	517	
Pseudomonas aeruginosa 2192	761.33	147.8	354	-
Pseudomonas aeruginosa PAO1	721.67	156.3	312	-
Pseudomonas aeruginosa UCBPP-PA14	742.22	148.2	340	-
Salmonella bongori 12149	496.78	191.8	320	
Salmonella enterica subsp. enterica serovar Choleraesuis str. SC-B67	525.56	182.1	308	-
Salmonella typhimurium LT2	522.78	181.0	302	-
Vibrio alginolyticus 12G01	512.89	170.0	555	
Vibrio cholerae AM-19226	447.00	165.2	324	
Vibrio splendidus 12B01	459.67	175.6	479	
Vibrio vulnificus YJ016	523.89	182.7	477	
Planctomycetes				
Pirellula sp. 1	720.89	199.3	621	
Spirochaetes				
Borrelia afzelii PKo	35.44	13.59	36	
Borrelia burgdorferi B31	118.11	136.5	28	
Leptospira interrogans serovar Copenhageni str. Fiocruz L1-130	231.00	59.13	212	
Treponema denticola ATCC 35405	154.88	30.97	124	
Treponema pallidum subsp. pallidum str. Nichols	100.9	22.29	195	+
Thermotogae				
Thermotoga maritima MSB8	173.78	56.39	133	
Thermotoga petrophila RKU-1	150.56	38.08	120	
LFRE2a				
	Mean Occ. of Random LFREs	95 % CI	Obs. Occ. of Actual LFREs	+/-
Actinobacteria-Actinomycetales				
Corynebacterium diphtheriae NCTC 13129	1.7	1.24	0	-
Corynebacterium efficiens YS-314	0.4	0.32	2	+
Mycobacterium bovis AF2122/97	0.6	0.67	1	

Mycobacterium tuberculosis CDC1551	0.5	0.60	1	
Streptomyces avermitilis MA-4680	0.1	0.20	0	
Streptomyces coelicolor A3(2)	0	0.00	0	
Alpha Proteobacteria				
Agrobacterium tumefaciens str. C58	2.7	2.73	5	
Bradyrhizobium japonicum USDA 110	2.8	2.81	3	
Brucella abortus biovar 1 str. 9-941	1.4	0.73	0	-
Brucella melitenus 16M	1.4	0.73	0	-
Ehrlichia canis str. Jake	0.7	0.78	12	+
Ehrlichia chaffeensis str. Arkansas	1.8	1.05	5	+
Ehrlichia ruminantium str. Gardel	2.4	1.90	2	
Nitrobacter hamburgensis X14	1.4	1.10	0	-
Rhizobium leguminosarum bv. viciae 3841	3.3	2.26	6	+
Rhodospirillum rubrum	1.1	1.19	2	
Rickettsia akari str. Hartford	1.1	1.53	4	+
Rickettsia rickettsii	1.3	1.13	4	+
Aquificae				
Aquifex aeolicus VF5	2	1.87	2	
Bacteriodetes				
Bacteroides fragilis NCTC 9343	4	2.32	6	
Bacteroides fragilis YCH46	4	2.00	5	
Bacteroides thetaiotaomicron VPI-5482	6.3	3.13	4	
Cytophaga hutchinsonii ATCC 33406	7.1	6.17	2	
Flavobacterium psychrophilum JIP02/86	3.8	1.59	5	
Beta Proteobacteria				
Bordetella bronchiseptica RB50	0.5	0.60	1	
Bordetella parapertussis 12822	0.3	0.30	2	+
Bordetella pertussis Tohama I	1	1.05	1	
Burkholderia ambifaria AMMD	1.3	1.10	1	
Burkholderia cenocepacia AU 1054	1.7	2.07	2	
Chromobacterium violaceum ATCC 12472	0.8	0.57	3	+
Neisseria gonorrhoeae FA 1090	0.2	0.26	0	
Neisseria meningitidis FAM18	0.2	0.26	0	
Nitrosomonas europaea ATCC	1.1	1.41	0	

19718				
Nitrosomonas eutropha C71	2.6	2.55	3	
Cyanobacteria				
Nostoc sp. PCC 7120	3.8	2.49	11	+
Prochlorococcus marinus str. MIT 9211	2.4	1.66	12	+
Synechococcus elongatus PCC 6301	0.9	1.07	2	
Synechococcus sp. CC9605	0.7	0.59	1	
Deinococcus-Thermus				
Deinococcus geothermalis DSM11300	0.1	0.20	0	
Deinococcus radiodurans R1	0.7	0.83	0	
Thermus thermophilus HB27	0.2	0.39	0	
Thermus thermophilus HB8	0.1	0.20	0	
Delta Proteobacteria				
Bdellovibrio bacteriovorus HD100	2.8	1.96	0	-
Desulfovibrio desulfuricans G20	0.8	1.00	0	
Desulfovibrio vulgaris subsp. vulgaris str. Hildenborough	1.1	1.15	0	
Desulfuromonas acetoxidans	1.3	1.40	3	+
Geobacter metallireducens GS-15	1.6	0.78	2	
Pelobacter carbinolicus DSM 2380	2.6	2.11	1	
Syntrophobacter fumaroxidans MPOB	2.8	1.96	0	-
Epsilon Proteobacteria				
Campylobacter jejuni subsp. doylei 269.97	4.3	6.31	8	
Campylobacter jejuni subsp. jejuni 260.94	3.9	5.95	7	
Campylobacter jejuni subsp. jejuni 81-176	3.3	4.97	5	
Helicobacter pylori 26656	3	3.20	8	+
Helicobacter pylori J99	2.8	2.99	8	+
Thiomicrospira crunogena XCL-2	3	2.77	5	
Wolinella succinogenes DSM 1740	2.6	2.57	1	
Firmicutes Bacilli				
Bacillus anthracis str. A1055	9.3	4.32	14	+
Bacillus anthracis str. 'Ames Ancestor'	10	4.77	14	
Bacillus cereus ATCC 10987	8.7	4.79	18	+
Bacillus cereus ZK	8.4	4.05	12	
Bacillus licheniformis ATCC	5.5	2.71	2	

14580				
Bacillus thuringiensis serovar konkukian str. 97-27	8.7	4.22	14	+
Enterococcus faecalis V583	5.6	3.22	3	
Lactobacillus acidophilus NCFM	3.8	2.98	0	-
Lactobacillus brevis ATCC 367	3.7	3.42	2	
Lactobacillus gasseri ATCC 33323	5.1	3.54	3	
Lactococcus lactis subsp. lactis Il1403	5	3.61	8	
Staphylococcus aureus RF122	3	2.17	2	
Staphylococcus haemolyticus JCSC1435	1.3	0.88	4	+
Streptococcus pneumoniae 23F	9.3	4.32	14	+
Streptococcus uberis 0140J	10	4.77	14	
Firmicutes Clostridium/Mollicutes				
Clostridium acetobutylicum ATCC 824	8	6.14	2	
Clostridium botulinum A str. ATCC 19397	9.2	10.95	24	+
Spiroplasma kunkelii CR2-3x	2.3	1.83	2	
Gamma Proteobacteria				
Acinetobacter baumannii ATCC 17978	4.8	2.86	6	
Azotobacter vinelandii	0.7	0.78	0	
Escherichia coli K12	3.2	1.67	2	
Escherichia coli O157:H7	4.2	1.80	0	-
Francisella tularensis subsp. holarctica FTA	2.4	2.41	4	
Francisella tularensis subsp. tularensis FSC198	2.3	2.23	4	
Haemophilus influenzae 86-028NP	2.7	1.65	3	
Legionella pneumophila str. Lens	3.7	2.61	5	
Methylococcus capsulatus str. Bath	1.4	0.98	0	-
Photobacterium profundum 3TCK	7.9	3.68	7	
Pseudomonas aeruginosa 2192	1.5	1.71	1	
Pseudomonas aeruginosa PAO1	1.6	1.88	1	
Pseudomonas aeruginosa UCBPP-PA14	1.3	1.52	1	
Salmonella bongori 12149	2.3	1.31	3	
Salmonella enterica subsp. enterica serovar Choleraesuis str. SC-B67	3.3	1.98	0	-
Salmonella typhimurium LT2	3.8	2.08	0	-
Vibrio alginolyticus 12G01	5.1	2.60	9	+

Vibrio cholerae AM-19226	3.2	1.65	2	
Vibrio splendidus 12B01	5.7	3.80	0	-
Vibrio vulnificus YJ016	4.4	2.65	2	
Planctomycetes				
Pirellula sp. 1	2.5	2.27	2	
Spirochaetes				
Borrelia afzelii PKo	1.5	1.35	2	
Borrelia burgdorferi B31	1.7	1.55	2	
Leptospira interrogans serovar Copenhageni str. Fiocruz L1-130	6.3	4.12	11	+
Treponema denticola ATCC 35405	5.7	3.91	1	-
Treponema pallidum subsp. pallidum str. Nichols	1.4	0.78	0	-
Thermotogae				
Thermotoga maritima MSB8	1.8	1.23	4	+
Thermotoga petrophila RKU-1	1.6	1.35	3	+
LFRE2b				
	Mean Occ. of Random LFREs	95 % CI	Obs. Occ. of Actual LFREs	+/-
Actinobacteria-Actinomycetales				
Corynebacterium diphtheriae NCTC 13129	7.3	3.68	12	+
Corynebacterium efficiens YS-314	1.9	1.00	5	+
Mycobacterium bovis AF2122/97	3.1	2.45	1	
Mycobacterium tuberculosis CDC1551	2.9	2.38	1	
Streptomyces avermitilis MA-4680	3.2	3.32	2	
Streptomyces coelicolor A3(2)	1.3	1.28	0	-
Alpha Proteobacteria				
Agrobacterium tumefaciens str. C58	23.2	24.34	18	
Bradyrhizobium japonicum USDA 110	8.4	8.05	5	
Brucella abortus biovar 1 str. 9-941	7.3	6.17	4	
Brucella melitenus 16M	7.6	6.24	4	
Ehrlichia canis str. Jake	7.0	4.95	9	
Ehrlichia chaffeensis str. Arkansas	13.9	11.04	4	
Ehrlichia ruminantium str. Gardel	8.1	4.36	4	
Nitrobacter hamburgensis X14	6.8	7.93	1	
Rhizobium leguminosarum bv. viciae 3841	15.4	16.39	5	

Rhodospirillum rubrum	5.5	5.98	1	
Rickettsia akari str. Hartford	6.7	5.01	2	
Rickettsia rickettsii	10.0	5.35	2	-
Aquificae				
Aquifex aeolicus VF5	7.6	2.81	4	
Bacteriodetes				
Bacteroides fragilis NCTC 9343	20.6	6.02	20	
Bacteroides fragilis YCH46	16.5	5.67	25	+
Bacteroides thetaiotaomicron VPI-5482	34.2	18.55	18	
Cytophaga hutchinsonii ATCC 33406	15.5	7.16	7	-
Flavobacterium psychrophilum JIP02/86	16.7	6.85	3	-
Beta Proteobacteria				
Bordetella bronchiseptica RB50	3.8	3.38	1	
Bordetella parapertussis 12822	3.6	3.11	0	-
Bordetella pertussis Tohama I	2.3	1.92	4	
Burkholderia ambifaria AMMD	3.8	3.59	1	
Burkholderia cenocepacia AU 1054	3.6	3.35	3	
Chromobacterium violaceum ATCC 12472	4.1	2.72	1	-
Neisseria gonorrhoeae FA 1090	3.3	2.63	1	
Neisseria meningitidis FAM18	3.9	2.96	2	
Nitrosomonas europaea ATCC 19718	10.5	5.07	2	-
Nitrosomonas eutropha C71	10.2	4.70	8	
Cyanobacteria				
Nostoc sp. PCC 7120	43.6	22.22	36	
Prochlorococcus marinus str. MIT 9211	12.6	4.92	9	
Synechococcus elongatus PCC 6301	4.2	1.80	12	+
Synechococcus sp. CC9605	3.1	2.33	8	+
Deinococcus-Thermus				
Deinococcus geothermalis DSM11300	1.2	0.91	6	+
Deinococcus radiodurans R1	2.5	2.34	8	+
Thermus thermophilus HB27	0.4	0.55	5	+
Thermus thermophilus HB8	0.2	0.28	6	+
Delta Proteobacteria				

Bdellovibrio bacteriovorus HD100	5.1	2.21	3	
Desulfovibrio desulfuricans G20	5.6	1.79	0	-
Desulfovibrio vulgaris subsp. vulgaris str. Hildenborough	4.3	5.22	5	
Desulfuromonas acetoxidans	3.9	4.04	4	
Geobacter metallireducens GS-15	5.8	3.26	5	
Pelobacter carbinolicus DSM 2380	5.4	5.41	5	
Syntrophobacter fumaroxidans MPOB	5.1	2.21	3	
Epsilon Proteobacteria				
Campylobacter jejuni subsp. doylei 269.97	11.0	7.67	15	
Campylobacter jejuni subsp. jejuni 260.94	13.8	14.29	9	
Campylobacter jejuni subsp. jejuni 81-176	15.0	14.33	10	
Helicobacter pylori 26656	7.0	6.27	2	
Helicobacter pylori J99	7.8	7.03	19	+
Thiomicrospira crunogena XCL-2	11.5	9.81	8	
Wolinella succinogenes DSM 1740	2.8	2.26	15	+
Firmicutes Bacilli				
Bacillus anthracis str. A1055	23.9	10.99	9	-
Bacillus anthracis str. 'Ames Ancestor'	25.4	10.99	9	-
Bacillus cereus ATCC 10987	37.7	22.43	8	-
Bacillus cereus ZK	23.5	11.32	10	-
Bacillus licheniformis ATCC 14580	8.8	5.20	9	
Bacillus thuringiensis serovar konkukian str. 97-27	29.5	10.72	6	-
Enterococcus faecalis V583	15.6	4.86	19	
Lactobacillus acidophilus NCFM	15.8	1.72	0	-
Lactobacillus brevis ATCC 367	9.4	4.54	11	
Lactobacillus gasseri ATCC 33323	13.7	8.14	10	
Lactococcus lactis subsp. lactis Il1403	14.2	6.79	11	
Staphylococcus aureus RF122	9.5	4.79	16	+
Staphylococcus haemolyticus JCSC1435	11.7	6.37	14	
Streptococcus pneumoniae 23F	23.9	10.99	9	-
Streptococcus uberis 0140J	25.4	10.99	9	-
Firmicutes Clostridium/Mollicutes				
Clostridium acetobutylicum ATCC	21.9	12.12	15	

824				
Clostridium botulinum A str. ATCC 19397	27.5	17.09	12	
Spiroplasma kunkelii CR2-3x	5.4	4.58	3	
Gamma Proteobacteria				
Acinetobacter baumannii ATCC 17978	21.2	7.73	17	
Azotobacter vinelandii	4.6	4.72	4	
Escherichia coli K12	12.2	3.87	3	-
Escherichia coli O157:H7	14.5	4.19	1	-
Francisella tularensis subsp. holarctica FTA	12.7	5.46	6	-
Francisella tularensis subsp. tularensis FSC198	12.9	5.98	6	-
Haemophilus influenzae 86-028NP	11.8	3.65	18	+
Legionella pneumophila str. Lens	16.7	10.17	10	
Methylococcus capsulatus str. Bath	4.1	3.61	2	
Photobacterium profundum 3TCK	26.5	10.66	11	-
Pseudomonas aeruginosa 2192	4.1	2.91	3	
Pseudomonas aeruginosa PAO1	4.1	3.88	1	
Pseudomonas aeruginosa UCBPP-PA14	5.3	5.18	1	
Salmonella bongori 12149	14.1	5.85	4	-
Salmonella enterica subsp. enterica serovar Choleraesuis str. SC-B67	14.6	5.69	3	-
Salmonella typhimurium LT2	13.6	5.41	0	-
Vibrio alginolyticus 12G01	25.2	8.47	18	
Vibrio cholerae AM-19226	12.7	6.34	9	
Vibrio splendidus 12B01	29.6	4.57	0	-
Vibrio vulnificus YJ016	15.0	4.07	10	-
Planctomycetes				
Pirellula sp. 1	11.4	8.37	8	
Spirochaetes				
Borrelia afzelii PKo	7.4	4.86	4	
Borrelia burgdorferi B31	10.0	5.73	12	
Leptospira interrogans serovar Copenhageni str. Fiocruz L1-130	23.6	13.82	20	
Treponema denticola ATCC 35405	17.2	13.40	5	
Treponema pallidum subsp. pallidum str. Nichols	2.5	1.71	2	
Thermotogae				
Thermotoga maritima MSB8	5.3	5.19	7	

Thermotoga petrophila RKU-1	4.4	3.77	5	
LFRE3				
	Mean Occ. of Random LFREs	95 % CI	Obs. Occ. of Actual LFREs	+/-
Actinobacteria-Actinomycetales				
Corynebacterium diphtheriae NCTC 13129	0.3	0.30	0	
Corynebacterium efficiens YS-314	0	0.00	0	
Mycobacterium bovis AF2122/97	0.1	0.20	0	
Mycobacterium tuberculosis CDC1551	0.1	0.20	0	
Streptomyces avermitilis MA-4680	0	0.00	0	
Streptomyces coelicolor A3(2)	0	0.00	0	
Alpha Proteobacteria				
Agrobacterium tumefaciens str. C58	0	0.00	0	
Bradyrhizobium japonicum USDA 110	0	0.00	0	
Brucella abortus biovar 1 str. 9-941	0	0.00	0	
Brucella melitenus 16M	0	0.00	0	
Ehrlichia canis str. Jake	0	0.00	0	
Ehrlichia chaffeensis str. Arkansas	0	0.00	0	
Ehrlichia ruminantium str. Gardel	0	0.00	0	
Nitrobacter hamburgensis X14	0	0.00	0	
Rhizobium leguminosarum bv. viciae 3841	0.1	0.20	0	
Rhodospirillum rubrum	0	0.00	0	
Rickettsia akari str. Hartford	0	0.00	0	
Rickettsia rickettsii	0.3	0.30	0	
Aquificae				
Aquifex aeolicus VF5	0.1	0.20	0	
Bacteriodetes				
Bacteroides fragilis NCTC 9343	0	0.00	0	
Bacteroides fragilis YCH46	0.1	0.20	0	
Bacteroides thetaiotaomicron VPI-5482	0.1	0.20	0	
Cytophaga hutchinsonii ATCC 33406	0.2	0.26	0	
Flavobacterium psychrophilum JIP02/86	0.2	0.26	0	
Beta Proteobacteria				

Bordetella bronchiseptica RB50	0	0.00	0	
Bordetella parapertussis 12822	0	0.00	0	
Bordetella pertussis Tohama I	0	0.00	0	
Burkholderia ambifaria AMMD	0.1	0.20	0	
Burkholderia cenocepacia AU 1054	0	0.00	0	
Chromobacterium violaceum ATCC 12472	0.1	0.20	0	
Neisseria gonorrhoeae FA 1090	0	0.00	0	
Neisseria meningitidis FAM18	0	0.00	0	
Nitrosomonas europaea ATCC 19718	0	0.00	0	
Nitrosomonas eutropha C71	0	0.00	0	
Cyanobacteria				
Nostoc sp. PCC 7120	0.4	0.43	0	
Prochlorococcus marinus str. MIT 9211	0	0.00	0	
Synechococcus elongatus PCC 6301	0	0.00	0	
Synechococcus sp. CC9605	0.1	0.20	0	
Deinococcus-Thermus				
Deinococcus geothermalis DSM11300	0	0.00	0	
Deinococcus radiodurans R1	0	0.00	0	
Thermus thermophilus HB27	0.1	0.20	0	
Thermus thermophilus HB8	0.1	0.20	0	
Delta Proteobacteria				
Bdellovibrio bacteriovorus HD100	0	0.00	0	
Desulfovibrio desulfuricans G20	0	0.00	0	
Desulfovibrio vulgaris subsp. vulgaris str. Hildenborough	0	0.00	0	
Desulfuromonas acetoxidans	0	0.00	0	
Geobacter metallireducens GS-15	0	0.00	0	
Pelobacter carbinolicus DSM 2380	0.2	0.26	0	
Syntrophobacter fumaroxidans MPOB	0	0.00	0	
Epsilon Proteobacteria				
Campylobacter jejuni subsp. doylei 269.97	0	0.00	0	
Campylobacter jejuni subsp. jejuni 260.94	0.1	0.20	0	
Campylobacter jejuni subsp. jejuni 81-176	0.1	0.20	0	

Helicobacter pylori 26656	0.1	0.20	0	
Helicobacter pylori J99	0	0.00	0	
Thiomicrospira crunogena XCL-2	0.1	0.20	0	
Wolinella succinogenes DSM 1740	0.1	0.20	0	-
Firmicutes Bacilli				
Bacillus anthracis str. A1055	0.2	0.26	0	
Bacillus anthracis str. 'Ames Ancestor'	0.1	0.20	0	
Bacillus cereus ATCC 10987	0.1	0.20	0	
Bacillus cereus ZK	0.1	0.20	0	
Bacillus licheniformis ATCC 14580	0	0.00	0	
Bacillus thuringiensis serovar konkukian str. 97-27	0	0.00	0	
Enterococcus faecalis V583	0.1	0.20	0	
Lactobacillus acidophilus NCFM	0.1	0.20	0	
Lactobacillus brevis ATCC 367	0.2	0.26	0	
Lactobacillus gasseri ATCC 33323	0	0.00	0	
Lactococcus lactis subsp. lactis Il1403	0.2	0.26	0	
Staphylococcus aureus RF122	0	0.00	0	
Staphylococcus haemolyticus JCSC1435	0	0.00	0	
Streptococcus pneumoniae 23F	0.2	0.26	0	
Streptococcus uberis 0140J	0.1	0.20	0	
Firmicutes Clostridium/Mollicutes				
Clostridium acetobutylicum ATCC 824	0.3	0.30	0	
Clostridium botulinum A str. ATCC 19397	0	0.00	0	
Spiroplasma kunkelii CR2-3x	0	0.00	0	
Gamma Proteobacteria				
Acinetobacter baumannii ATCC 17978	0.1	0.20	0	
Azotobacter vinelandii	0.1	0.20	0	
Escherichia coli K12	0.3	0.30	0	
Escherichia coli O157:H7	0.2	0.26	0	
Francisella tularensis subsp. holarctica FTA	0	0.00	0	
Francisella tularensis subsp. tularensis FSC198	0	0.00	0	
Haemophilus influenzae 86-028NP	0.1	0.20	0	
Legionella pneumophila str. Lens	0.2	0.26	0	

Methylococcus capsulatus str. Bath	0.1	0.20	0	
Photobacterium profundum 3TCK	0.7	0.59	0	-
Pseudomonas aeruginosa 2192	0.1	0.20	0	
Pseudomonas aeruginosa PAO1	0.2	0.26	0	
Pseudomonas aeruginosa UCBPP-PA14	0	0.00	0	
Salmonella bongori 12149	0.2	0.39	0	
Salmonella enterica subsp. enterica serovar Choleraesuis str. SC-B67	0	0.00	0	
Salmonella typhimurium LT2	0.1	0.20	0	
Vibrio alginolyticus 12G01	0.3	0.30	0	
Vibrio cholerae AM-19226	0.1	0.20	0	
Vibrio splendidus 12B01	0.3	0.42	0	
Vibrio vulnificus YJ016	0.5	0.33	0	
Planctomycetes				
Pirellula sp. 1	0	0.00	0	
Spirochaetes				
Borrelia afzelii PKo	0	0.00	0	
Borrelia burgdorferi B31	0.1	0.20	0	
Leptospira interrogans serovar Copenhageni str. Fiocruz L1-130	0.2	0.26	0	
Treponema denticola ATCC 35405	0.1	0.20	0	
Treponema pallidum subsp. pallidum str. Nichols	0.1	0.20	0	
Thermotogae				
Thermotoga maritima MSB8	0	0.00	0	
Thermotoga petrophila RKU-1	0	0.00	0	

(1) The number of LFRE consensus sequences in each genome was compared to the expected frequency (95 % confidence interval) of sequences of the same length and base composition in a given genome. LFREs that occurred at a greater or lower than expected frequency were marked with a (+) or (-), respectively.

Table A4.3: Summary of the occurrence of lactoferrin binding sites in comparison to expected frequencies[1] by species

	LFREs				
Species	**1a**	**1b**	**2a**	**2b**	**3**
Actinobacteria- Actinomycetales					
Corynebacterium diphtheriae NCTC 13129			-	+	
Corynebacterium efficiens YS-314	+	-	+	+	

Mycobacterium bovis AF2122/97	+	-			
Mycobacterium tuberculosis CDC1551	+	-			
Streptomyces avermitilis MA-4680					
Streptomyces coelicolor A3(2)				-	
Alpha Proteobacteria					
Agrobacterium tumefaciens str. C58	+	-			
Bradyrhizobium japonicum USDA 110		-			
Brucella abortus biovar 1 str. 9-941			-		
Brucella melitenus 16M		-	-		
Ehrlichia canis str. Jake			+		
Ehrlichia chaffeensis str. Arkansas		+	+		
Ehrlichia ruminantium str. Gardel	-	+			
Nitrobacter hamburgensis X14		-	-		
Rhizobium leguminosarum bv. viciae 3841		-	+		
Rhodospirillum rubrum		-			
Rickettsia akari str. Hartford			+		
Rickettsia rickettsii			+	-	
Aquificae					
Aquifex aeolicus VF5					
Bacteriodetes					
Bacteroides fragilis NCTC 9343					
Bacteroides fragilis YCH46		+		+	
Bacteroides thetaiotaomicron VPI-5482	+	+			
Cytophaga hutchinsonii ATCC 33406	+			-	
Flavobacterium psychrophilum JIP02/86	+			-	
Beta Proteobacteria					
Bordetella bronchiseptica RB50		-			
Bordetella parapertussis 12822	+		+	-	
Bordetella pertussis Tohama I		+			
Burkholderia ambifaria AMMD		+			
Burkholderia cenocepacia AU 1054		+			
Chromobacterium violaceum ATCC 12472		-	+	-	
Neisseria gonorrhoeae FA 1090	+				

Neisseria meningitidis FAM18					
Nitrosomonas europaea ATCC 19718				-	
Nitrosomonas eutropha C71					
Cyanobacteria					
Nostoc sp. PCC 7120	+	-	+		
Prochlorococcus marinus str. MIT 9211	+	-	+		
Synechococcus elongatus PCC 6301	-	-		+	
Synechococcus sp. CC9605		-		+	
Deinococcus-Thermus					
Deinococcus geothermalis DSM11300	+	-		+	
Deinococcus radiodurans R1	+			+	
Thermus thermophilus HB27		-		+	
Thermus thermophilus HB8		-		+	
Delta Proteobacteria					
Bdellovibrio bacteriovorus HD100			-		
Desulfovibrio desulfuricans G20		-		-	
Desulfovibrio vulgaris subsp. vulgaris str. Hildenborough	+				
Desulfuromonas acetoxidans		-	+		
Geobacter metallireducens GS-15		-			
Pelobacter carbinolicus DSM 2380		-			
Syntrophobacter fumaroxidans MPOB			-		
Epsilon Proteobacteria					
Campylobacter jejuni subsp. doylei 269.97	-	-			
Campylobacter jejuni subsp. jejuni 260.94	-				
Campylobacter jejuni subsp. jejuni 81-176	-	-			
Helicobacter pylori 26656		-	+		
Helicobacter pylori J99	-	-	+	+	
Thiomicrospira crunogena XCL-2		-			
Wolinella succinogenes DSM 1740	-	-		+	-
Firmicutes Bacilli					
Bacillus anthracis str. A1055	+		+	-	
Bacillus anthracis str. 'Ames Ancestor'	+			-	

Bacillus cereus ATCC 10987	+		+	-	
Bacillus cereus ZK	+			-	
Bacillus licheniformis ATCC 14580		-			
Bacillus thuringiensis serovar konkukian str. 97-27	+		+	-	
Enterococcus faecalis V583					
Lactobacillus acidophilus NCFM	-		-	-	
Lactobacillus brevis ATCC 367					
Lactobacillus gasseri ATCC 33323					
Lactococcus lactis subsp. lactis Il1403					
Staphylococcus aureus RF122	-			+	
Staphylococcus haemolyticus JCSC1435			+		
Streptococcus pneumoniae 23F	+		+	-	
Streptococcus uberis 0140J	+			-	
Firmicutes Clostridium/Mollicutes					
Clostridium acetobutylicum ATCC 824					
Clostridium botulinum A str. ATCC 19397	-		+		
Spiroplasma kunkelii CR2-3x	-	+			
Gamma Proteobacteria					
Acinetobacter baumannii ATCC 17978					
Azotobacter vinelandii		-			
Escherichia coli K12	+			-	
Escherichia coli O157:H7	+		-	-	
Francisella tularensis subsp. holarctica FTA	+	-		-	
Francisella tularensis subsp. tularensis FSC198	+	-		-	
Haemophilus influenzae 86-028NP	-			+	
Legionella pneumophila str. Lens		-			
Methylococcus capsulatus str. Bath		-	-		
Photobacterium profundum 3TCK				-	-
Pseudomonas aeruginosa 2192		-			
Pseudomonas aeruginosa PAO1		-			
Pseudomonas aeruginosa UCBPP-PA14		-			
Salmonella bongori 12149				-	

Salmonella enterica subsp. enterica serovar Choleraesuis str. SC-B67		-	-	-	
Salmonella typhimurium LT2		-	-	-	
Vibrio alginolyticus 12G01			+		
Vibrio cholerae AM-19226					
Vibrio splendidus 12B01			-	-	
Vibrio vulnificus YJ016				-	
Planctomycetes					
Pirellula sp. 1					
Spirochaetes					
Borrelia afzelii PKo	-				
Borrelia burgdorferi B31	-				
Leptospira interrogans serovar Copenhageni str. Fiocruz L1-130	-		+		
Treponema denticola ATCC 35405			-		
Treponema pallidum subsp. pallidum str. Nichols	+	+	-		
Thermotogae					
Thermotoga maritima MSB8			+		
Thermotoga petrophila RKU-1			+		

(1) The number of LFRE consensus sequences in each genome was compared to the expected frequency (95 % confidence interval) of sequences of the same length and base composition in a given genome. LFREs that occurred at a greater or lower than expected frequency were marked with a (+) or (-), respectively.

Table A4.4: Distance between features near and downstream of lactoferrin binding sites in the *H. pylori* strain J99 genome

Nearest Feature	Distance to Nearest Feature (bp)[1]	Distance to Nearest Downstream Feature (bp)[1]
LFRE1a		
outer membrane protein - adhesin	1312	-1998
LFRE1b		
putative vacuolating cytotoxin (VacA) paralog	3213	-4039
DNA-directed RNA polymerase beta subunit (EC 2.7.7.6) / DNA-directed RNA polymerase beta' subunit (EC 2.7.7.6)	2649	-6111

putative	2046	NA
putative Outer membrane protein	1996	-1731
Flagellar hook-associated protein flgL	1779	-2022
putative Outer membrane protein	1509	-258
Multi antimicrobial extrusion protein (Na(+)/drug antiporter), MATE family of MDR efflux pumps	1257	-148
[NiFe] hydrogenase metallocenter assembly protein HypF	833	-1443
Leucyl-tRNA synthetase (EC 6.1.1.4)	777	-1640
INTEGRASE/RECOMBINASE (XERCD FAMILY)	718	-4755
UDP-glucose 4-epimerase (EC 5.1.3.2)	699	-1614
Excinuclease ABC subunit C	658	-1136
Phosphogluconate dehydratase (EC 4.2.1.12)	654	-720
Membrane-fusion protein	652	-349
Polyphosphate kinase (EC 2.7.4.1)	648	-1418
tRNA uridine 5-carboxymethylaminomethyl modification enzyme gidA	591	-687
Ribonuclease BN (EC 3.1.-.-)	558	-4466
Type III restriction-modification system DNA endonuclease res (EC 3.1.21.5)	546	-955
Translation elongation factor P @ Translation initiation factor 5A	490	-620
putative	484	-152
Flagellar P-ring protein flgI	433	-619
putative oxidoreductase	411	-4031
putative transporter	382	-478
Aspartate aminotransferase (EC 2.6.1.1)	372	-967
outer membrane protein - adhesin	349	-3078
Dipeptide transport system permease protein dppC (TC 3.A.1.5.2)	346	-244
putative	346	-404
putative periplasmic protein	336	-989
Exodeoxyribonuclease III (EC 3.1.11.2)	273	-1539
Purine nucleoside phosphorylase (EC 2.4.2.1)	264	-900
Acetone carboxylase, beta subunit (EC 6.4.1.6) / N-methylhydantoinase A (EC 3.5.2.14)	201	-1949
putative	192	-265
Two-component system response regulator	169	-278
putative TRANSCRIPTIONAL REGULATOR	169	-2450

2-keto-3-deoxy-D-arabino-heptulosonate-7-phosphate synthase II (EC 2.5.1.54) # AroA II	142	-323
Acyl carrier protein	67	-272
putative	-43	
Acetone carboxylase, gamma subunit (EC 6.4.1.6)	-151	
Lipoprotein releasing system transmembrane protein LolC	-154	
Polysaccharide biosynthesis protein WlaX	-295	
putative	-427	
Diaminopimelate decarboxylase (EC 4.1.1.20)	-1586	
LFRE2a		
Transcription-repair coupling factor	2392	-738
Flagellar M-ring protein fliF	1537	-183
Phospholipid-lipopolysaccharide ABC transporter	1033	-616
putative	943	-79
probable chlorohydrolase	502	-974
putative	226	-591
Flagellar basal-body rod protein flgG	-169	
Putative predicted metal-dependent hydrolase	-523	
LFRE2b		
Flagellar M-ring protein fliF	1423	-1063
Phospholipid-lipopolysaccharide ABC transporter	1315	NA
DNA gyrase subunit A (EC 5.99.1.3)	1132	-321
cyclopocyclopropane fatty acid synthase	1080	NA
Mannose-6-phosphate isomerase (EC 5.3.1.8) / Mannose-1-phosphate guanylyltransferase (GDP) (EC 2.7.7.22)	973	-1047
ADP-L-glycero-D-manno-heptose-6-epimerase (EC 5.1.3.20)	895	-978
Predicted D-lactate dehydrogenase, Fe-S protein, FAD/FMN-containing	763	-536
3-polyprenyl-4-hydroxybenzoate carboxy-lyase (EC 4.1.1.-)	625	-322
putative periplasmic protein	538	-4598
S-adenosyl-methyltransferase mraW (EC 2.1.1.-)	502	-797

TETRATRICOPEPTIDE REPEAT FAMILY PROTEIN	430	NA
putative periplasmic protein	354	-591
Methylated-DNA--protein-cysteine methyltransferase (EC 2.1.1.63)	276	-2045
Fructose-bisphosphate aldolase class II (EC 4.1.2.13)	271	-344
Acetone carboxylase, alpha subunit (EC 6.4.1.6) / N-methylhydantoinase B	259	-291
putative keto-acid dehydrogenase	241	-992
LSU ribosomal protein L6p (L9e)	222	-2997
MOLYBDOPTERIN BIOSYNTHESIS PROTEIN	145	-422
Potassium efflux system kefA / Small-conductance mechanosensitive channel	-237	
CDP-diacylglycerol pyrophosphatase (EC 3.6.1.26)	1423	-1063
putative	1315	NA

(1) Position of lactoferrin in relationship to ORFs in bp, either downstream the nearest ORF or upstream (-).

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